University of Alberta

IDENTIFICATION OF NOVEL WILMS' TUMOR RELATED GENES BY USING DIFFERENTIAL DISPLAY POLYMERASE CHAIN REACTION (DD-PCR)

By

Hong Chen ©

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Chen Hong

Permanent address:
MOP
Cross Cancer Institute
11560 University Ave
Edmonton AB T6G 1Z2

Date: Sept 11 97

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled IDENTIFICATION OF NOVEL WILMS' TUMOR RELATED GENES BY USING DIFFERENTIAL DISPLAY POLYMERASE CHAIN REACTION (DD-PCR) submitted by Hong Chen in partial fulfillment of the requirements for the degree of Master of Science in Medical Sciences-Oncology.

Paul E. Grundy

Dr. Paul E. Grundy (supervisor)

Roseline Godbout

Dr. Roseline Godbout

Heather M. Dermid

Dr. Heather McDermid

Dr. Joan Allalunis Turner

Date: Sept 11/97

To my parents and my husband

ABSTRACT

Wilms' tumor is one of the most common childhood solid tumors. The WT1 gene on chromosome 11p13 is the only identified and characterized Wilms' tumor gene. Genomic imprinting in chromosomal region 11p15 and LOH on other chromosomal loci are also involved in some of the tumor development. Wilms' tumor is generally successfully treated with a long-term survival rate in excess of 85%. Further refinement of the treatment will depend largely on the identification of novel prognostic factors.

Previous molecular studies revealed a tumor specific loss of chromosome 16q which was associated with poor outcome in about 20% Wilms' tumors, suggesting a putative tumor related genes at this locus, most possibly having prognostic significance.

To identify putative WT gene(s), we used the DD-PCR technique to compare two categories of Wilms' tumors chosen on the basis of certain selective criteria. 30 DD-PCR reactions yielded 23 differential displayed fragments. One fragment whose differential expression in Wilms' tumor was confirmed revealed no similarity to any known human genes. In addition, this fragment was located to human chromosome 3. More DD-PCR reactions and further characterization of the identified DD-fragments are needed to identify novel Wilms' tumor related genes.

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ABBREVIATIONS

APS	ammonium persulfate
β-ME	β-mercaptoethanol
bp	base pairs
BSA	bovine serum albumin
BWS	Beckwith-Wiedemann syndrome
CAT	erythrocyte catalase
cDNA	complementary deoxyribonucleic acid
Ci	curie
cpm	counts per minute
DD-PCR	differential display polymerase chain reaction
DMF	dimethyl formamide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid, disodium salt
EGR-I	early growth response factor 1
EtBr	ethidium bromide
FH	favorable histology
FSB	first strand buffer
FSHB	β subunit of follicle-stimulating hormone
FWT 1	familial Wilms' tumor gene 1
hr.	hour

IGF-2	insulin like growth factor type 2
ILNR	intralobar nephrogenic rests
kb	kilo-base pairs
KTS	three amino acids, lysine, threonine and serine, which are involved in the alternative splice II in WT1 gene
LB medium	Luria-Bertani medium
LOH	loss of heterozygosity
LOI	loss of genomic imprinting
µg	microgram
µl	micro liter
min.	minute
mRNA	messenger ribonucleic acid
MOPS	3-[N-Morpholino]propane-sulphonic acid
NWTS	National Wilms' Tumor Study
O/N	overnight
PAGE	polyacrylamide gel electrophoresis
PDGF-A	platelet-derived growth factor A chain
PLNR	perilobar nephrogenic rests
PVP	polyvinylpyrrolidone
RFLP	restrictive fragment length polymorphism
RT-PCR	reverse transcription polymerase chain reaction
TEMED	N,N,N,N,-tetramethylenediamine

TK	thymidine kinase
UH	unfavorable histology
WAGR syndrome	Wilms' tumor, Aniridia, Genitourinary anomalies, mental Retardation
WT	Wilms' tumor
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER I INTRODUCTION

I. Wilms' tumor

I.1. General background

Wilms' tumor, or nephroblastoma, is an embryonal malignancy of the kidney. With an incidence of more than one in 10,000 children under the age of 15 (1,2), it is one of the most common childhood solid tumors, ranking below central nervous system tumors, lymphoma, neuroblastoma and soft tissue sarcoma (3). Though occurring very rarely in the neonatal period (4), over three quarters of Wilms' tumors afflict children younger than four years old, with a peak incidence at three years of age. At least 90% of the patients are less than seven years old when diagnosed (5). There is no gender-specific distribution among the afflicted patients (4). Wilms' tumor is very rare in the adult population. At this time, only about 200 cases of adult Wilms' tumor have been reported (6,7,8,9). Due to the lack of a consistent pathological nomenclature used in the past, only a few of these latter diagnoses can be verified.

Most Wilms' tumors occur sporadically and only about 1% of the patients have family histories. Wilms' tumor can appear unilaterally, bilaterally or multifocally in a patient. The majority of tumors are sporadic and unilateral lesions with only 5-10% of cases reported as bilateral. Compared to unilateral tumors, bilateral tumors are more likely to occur at an earlier age and with a higher likelihood of having other congenital disorders. According to a National Wilms' Tumor Study in 1988 (5), the median ages at diagnosis for males and females with unilateral Wilms' tumors were 36.5 months and 42.5 months,

respectively. Those with bilateral tumors had an earlier age of diagnosis at 23.5 months for males and 30.5 months for females. Patients with aniridia or the characteristic genitourinary (GU) anomalies were much younger than Wilms' tumor patients without those associated anomalies (5).

Unlike most childhood tumors which are independent of other diseases, Wilms' tumor has the striking feature of being associated with many congenital anomalies such as aniridia, genitourinary anomalies (GU) and mental retardation (10). Wilms' tumor was first found to be associated with bilateral aniridia in the 1960s and this association was expanded to include GU anomalies and mental retardation. Aniridia refers to the absence or malformation of the iris. Its incidence in Wilms' tumor patients is about 1 in 70, a rate that is about 1,000 times higher than that in the general population. Wilms' tumor occurs in roughly one third of children having aniridia. GU anomalies which involve the kidney, collecting systems, internal and external genitalia are more frequently found in patients with bilateral Wilms' tumor. The association of Wilms' tumor, aniridia, GU anomalies and mental retardation has led to the acronym WAGR syndrome.

Patients with Beckwith-Wiedemann syndrome (BWS) are also at an increased risk of developing Wilms' tumor and other embryonal tumors (11,12). BWS is a WT-associated syndrome characterized by umbilical hernia, macroglossia, neonatal hypoglycemia and giantism. The prevalence of BWS is about 7 per 100,000 births. About 5-10% of the patients will develop Wilms' tumor, adrenocortical carcinoma or hepatoblastoma. Another WT-associated syndrome is Denys-Drash syndrome (DDS) (13,14). DDS refers to the

association of Wilms' tumor, intersex disorders and renal nephropathy. Children with DDS have severe nephropathy which will lead to progressive renal failure and external genital abnormalities. More than 70% of DDS sufferers develop Wilms' tumor. In addition, Wilms' tumor is associated with other congenital developmental anomalies like Perlman syndrome (nephroblastomatosis and genital abnormalities) (15), and Klippel-Trenaunay syndrome (cutaneous hemangiomas, bone and soft tissue hypertrophy) (16).

I.2. Pathological features

Wilms' tumor normally displays a triphasic histology containing varying amounts of blastemal, epithelial and stromal cell types. The malignant transformation in Wilms' tumor is thought to originate in cells of the metanephric blastema (undifferentiated intermediate mesoderm) of the fetal embryo which are programmed to differentiate and develop into the kidney. The mammalian urogenital system develops through three stages forming three sets of kidney: pronephros, mesonephros and metanephros (permanent kidney), respectively. All three steps are characterized by the induction of a mesenchymal to epithelial transformation (17,18). The basic functional units of the kidney, nephrons, are formed from the metanephric blastema in the third stage. This formation continues until the late stages of intrauterine life. Normally, in the tenth fetal month, the metanephrogenic tissue disappears, suggesting that the postnatal formation of nephrons does not occur (19). The metanephrogenic blastemal cells are referred to as nephrogenic rests if they persist in the newborn. These rests

are observed in approximately 1% of all the infants autopsied and considered to represent incompletely differentiated nephrogenic cells in which neoplastic transformation can occur, since approximately one third of Wilms' tumor patients have been found to have this lesion (5,20). The incidence of nephrogenic rests is much higher than that of Wilms' tumor in the general population probably because most of these rests do not progress or undergo spontaneous regression after birth. Based on the site of origin in the kidney, the histology and distribution, nephrogenic rests can be divided into intralobar nephrogenic rests (ILNR) and perilobar nephrogenic rests (PLNR). ILNR which are randomly distributed throughout the renal lobe have a prominent stromal component. PLNR are located at the periphery of the developing renal lobe, with a high composition of blastemal cells (20,21). Wilms' tumors associated with ILNR tend to mimic the entire spectrum of renal development, whereas those associated with PLNR tend to mimic only the late stages.

The National Wilms' Tumor Study (NWTS) has developed guidelines for the classification and treatment of Wilms' tumors. Generally, Wilms' tumors can be divided into favorable histology and unfavorable histology (22). According to the first NWTS, Wilms' tumor patients with a mixed histology of blastema, epithelial differentiation and more differentiated stromal elements had a favorable outlook. This tumor type accounted for more than 85% of all Wilms' tumor cases and was classified as favorable histology. The remaining 10-15% of tumors including clear cell sarcoma, rhabdoid tumor and anaplasia had a poorer outlook and were grouped as unfavorable histology. In the subsequent NWTS

trials (23), clear cell sarcoma of the kidney and rhabdoid tumor were excluded as variants of WT but considered to be separate neoplastic entities. Therefore, the anaplastic form of Wilms' tumor became the only form that could be placed in the unfavorable histology category. Histologically, anaplasia is characterized by the presence of nuclear giantism with multipolar mitotic figures. Present in only about 5% of WT patients, this form of tumor has been associated with a significantly worse outcome. In NWTS trials, the presence or absence of anaplastic cells in Wilms' tumor is enough to determine whether the tumor is placed into the "unfavorable" or "favorable" category.

The advent of chemotherapy in the 1960s, combined with radiotherapy and more aggressive surgical treatment of metastatic cases, has made Wilms' tumor one of the most successfully treated neoplastic diseases. Current survival rate for patients with tumors having favorable histology is more than 85% (22).

I.3. Genetic pathogenesis

The etiology of Wilms' tumor is not well known yet. However, the very early age of onset and the high incidence of association with congenital disorders suggest at least a partial genetic basis of the disease.

I.3.1. WT1 gene at 11p13

I.3.1.1. Isolation of WT1 gene

As early as in 1960, Miller and co-workers (10) reported an association between Wilms' tumor and bilateral aniridia (absence or malformation of iris).

They noted that about 1 in 70 Wilms' tumor patients had aniridia, a rate which was much higher than its incidence of 1 in 70,000 in the general population. Wilms' tumors occurred in approximately one third of aniridia patients, and in this setting had a greater incidence of bilateral involvement than did WT patients in general (10,24). This WT-aniridia association was then expanded to include genitourinary anomalies and mental retardation. Genitourinary anomalies included renal hypoplasia, unilateral renal agenesis, horseshoe kidneys, ureteral atresia, misplaced external penile urinary orifice and undescended testis (1, 10). These associated disorders were grouped as the WAGR syndrome.

Chromosome analysis of WAGR syndrome patients revealed a constitutional 11p deletion (25), which was the first clue suggesting possible involvement of chromosome 11p in the WAGR syndrome. Cytogenetic and molecular analysis further defined a WAGR locus at 11p13, a region that was thought to be associated with Wilms' tumor (26,27,28). Subsequent studies in sporadic Wilms' tumors confirmed the tumor-specific loss of genetic material on chromosome 11p (29,30,31,32,33). Loss of heterozygosity (LOH) at 11p occurred in about 50% of Wilms' tumor cases. This evidence strongly suggested the existence of a gene (or genes) on the short arm of chromosome 11 which was important in Wilms' tumor initiation or development.

After localizing a putative Wilms' tumor locus to chromosome 11p by cytogenetic and molecular studies in Wilms' tumor patients, the main objective of later studies was to isolate corresponding Wilms' tumor gene(s) at this locus. The successful strategy was the one proposed by Dr. Rose and colleagues in early 1990s which isolated random 11p13 DNA clones and localized them to the smallest 11p13 region that was commonly deleted in a series of Wilms' tumors.

In fact, the isolation of the first Wilms' tumor gene was not the success of a single laboratory but the culmination of many years of effort in many

laboratories and involved both the generation of somatic cell hybrids and the analysis of patients with chromosomal deletions and translocations. An crucial step in 11p13 mapping was the establishment of hybrid cell lines that contained the short arm of chromosome 11 as their only human DNA in a hamster cell background (34), which was based upon analysis of the closest DNA markers flanking the WAGR region: genes encoding erythrocyte catalase (CAT) and the β subunit of follicle-stimulating hormone (FSHB) (26,27). The development of these hybrid cell lines allowed segregation of the deleted chromosome 11 from its normal counterpart, providing valuable mapping reagents. Furthermore, a substantial number of 11p13 DNA markers had been isolated from chromosome 11-specific DNA libraries (35,36,37). Among them, D11S87 had been characterized as a WT marker and used to select WT specific cell lines in which it was homozygously deleted. Taking advantage of all these achievements, Rose and colleagues (38,39) completed a physical map of the 11p13 region. By working on a sporadic WT with deletions within 11p13, they limited the Wilms' tumor locus to a region of less than 345 kb which was homozygously deleted in the tumor. Using a library of human genomic DNA derived from a somatic cell hybrid, clones that were homozygously deleted in this tumor were identified. One clone within the smallest common region of deletion was then used to screen cDNA libraries derived from human embryonic kidney, human adult kidney and human pre-B cells. One cDNA clone WT33, which detected an approximately 3 kb transcript expressed in fetal kidney, spleen and some human leukemia cell lines, was isolated. Another clone, LK15, was also isolated independently by a group who used a chromosome jumping cloning technique (40). LK15 was found to detect the same size transcript as that of WT33 with a similar expression pattern. Both cDNAs were noted to have the highest expression levels in the embryonic kidney. The polypeptide structure predicted from WT33 sequence

had four zinc finger domains and a proline/glutamine-rich domain, suggesting that it might function as a transcription factor. The genetic location at 11p13, the tissue-specific expression and the putative transcription factor function supported the identity of these cDNAs as the Wilms' tumor gene at 11p13. This gene was named WT1 and considered to be a tumor suppressor gene responsible for Wilms tumorigenesis.

I.3.1.2. WT1 gene structure and transcripts

The WT1 gene consists of ten exons and spans about 50 kb (Figure 1.) (41,42,43). Two exons (exons 5 and 9) are alternatively spliced to yield four mRNA species, while each of the last 4 exons (exons 7 to 10) contains one zinc finger motif. The WT1 transcript is 3 kb, with a predicted polypeptide of 46-49 kDa reflecting the presence or absence of two alternative splices.

One alternative splicing event (splice I) can insert or remove exon 5 which encodes 17 amino acids. Another event (splice II) occurs within the terminal 9 nucleotides of exon 9, resulting in the insertion or deletion of three amino acids, lysine, threonine and serine (KTS) between exons 9 and 10 which encode zinc fingers 3 and 4, respectively. As a result, four distinct WT1 transcripts are formed:

splice form A: -51 bp/-9 bp (removing both exon 5 and KTS)

splice form B: +51 bp/-9 bp (inserting exon 5, deleting KTS)

splice form C: -51 bp/+9 bp (removing exon 5, inserting KTS)

splice form D: +51 bp/+9 bp (inserting both exon 5 and KTS)

All four WT1 isoforms of constant relative ratio are found in tissues expressing WT1, both in human and in mouse (42). In normal kidney tissue and Wilms' tumors, splice form D is 5 to 10-fold more prevalent than form A while forms B and C are intermediate in prevalence. Studies of the alternative splicing in

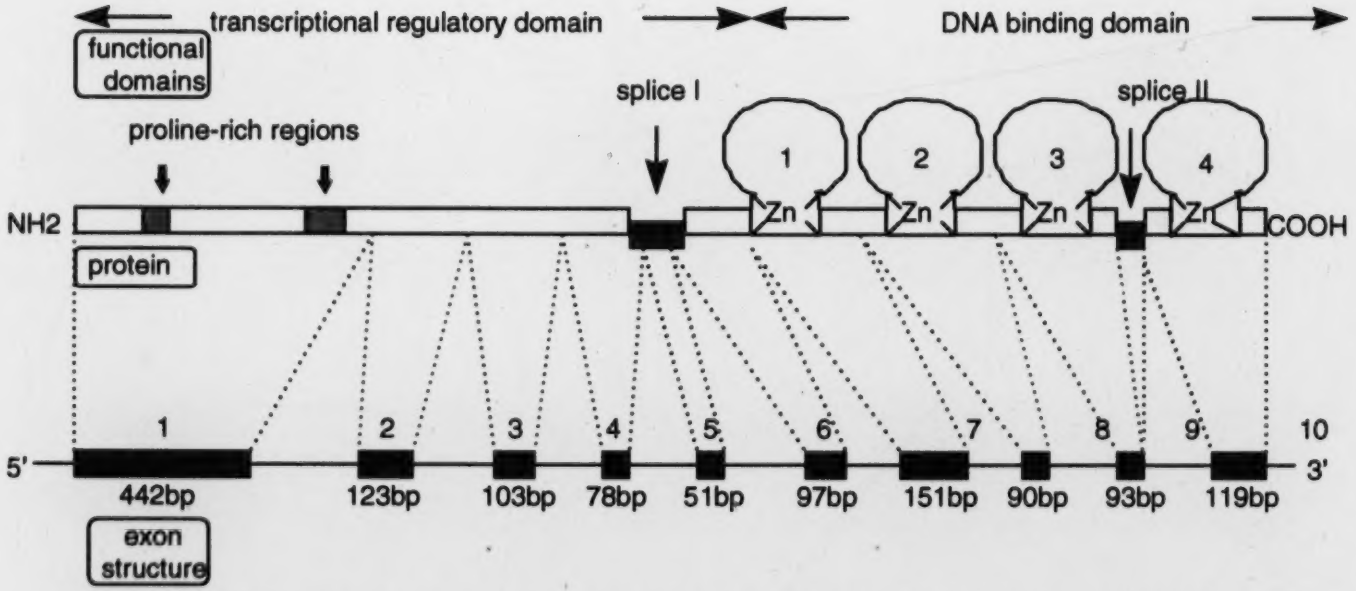


Figure 1. WT1 gene: exon structure, transcript and functional domains. (A) WT1 gene consists of 10 exons and spans about 50kb. Exon 5 and 9 are alternatively spliced . Each of the last 4 exons (7-10) has one zinc finger motif. (B) Predicted WT1 product contains one transcription regulatory domain and DNA binding domain which which contains 4 zinc finger structures (43).

Wilms' tumor have yielded little information on the functional importance of these different isoforms. Alternative splicing of exon 5 has been found to be disrupted in Wilms' tumors (44). The WT1 product with exon 5 can inhibit the progression of cells through the cell cycle, and this inhibition can be overcome by increasing the amount of activated cyclin/CDK complexes in the cell (45). Combined with the finding that the level of WT1 product with exon 5 isoform is decreased in Wilms' tumors (44), these observations suggest that loss of exon 5 may increase the proliferation capacity of these tumor cells. As discussed later, though splice II inserts only three amino acids between zinc fingers 3 and 4, alternative splicing at this site can affect the binding of WT1 to DNAs. The significance of alternative splicings is still under investigation.

The presence of zinc finger domains and a proline-rich domain in the WT1 gene product suggested a possible transcription regulatory function for WT1. This putative function has been confirmed by in vitro transient transformation assays. In various transient transformation assays, WT1 is shown to be either a negative or positive regulator of transcription (46,47,48). Since the WT1 product can bind the DNA sequence 5'GCGGGGGCG3' which was a recognition motif similar to the early growth response factor 1 (EGR-I) binding site (49,50), Madden and colleagues (46) used a reporter with EGR-I binding sites positioned in the thymidine kinase (TK) promoter and demonstrated that WT1 could repress transcription. WT1 was also found to repress the expression of several other genes promoting cell growth: insulin-like growth factor type 2 (IGF 2) (51) and the gene for platelet-derived growth factor A chain (PDGF-A) (52,53). The above assays also showed that WT1 exerts its function by binding to the regulatory regions of these genes. In Madden's experiment (46), the repressive ability of WT1 was proportional to the number of binding sites and was independent of the location of these sites (47). However, in a different

assay system from Wang's lab (48), location of the binding sites did affect WT1 activity. If the sites were present either upstream or downstream of the transcription start site, WT1 activated transcription; if the sites were both upstream and downstream to the start site, WT1 repressed transcription. Though no consistent results have been obtained and no exact mechanism is postulated, these observations support the concept that WT1 can affect transcription of some genes related to cell growth and possibly result in the imbalanced cell growth prone to neoplastic transformation. As discussed, the transcription regulating function of the WT1 gene mainly depends on its binding to other genes, any factors that alter its binding motif may affect its function. This may underlie the need for alternative splicing between zinc fingers 3 and 4 and may explain how WT1 isoforms function differentially in transcriptional regulation. The WT1 isoform with or without KTS has been found to have different binding motifs. The WT1 isoform without KTS(WT1-KTS) binds a DNA motif similar to EGR-I (49), represses transcription from the promoters of the EGR-I (46), PDGF-A (52), IGF-2 (51), retinoic acid receptor-α (54), PAX2 (55) and the IGF I receptor (56). WT1+KTS isoform represents nearly 80% of the WT1 transcripts and binds DNA motifs similar to WT1-KTS, but no consensus motif has been found (57). Since alternative splicing between zinc fingers 3 and 4 (splice II) occurs in the DNA binding domain, it might alter WT1 function by changing the binding motifs of WT1 products. By identifying different DNA binding motifs for different WT1 splice isoforms, more potential targets of WT1 may be found and thus may reveal how WT1 regulates cell growth and differentiation.

I.3.1.3. WT1 expression

The WT1 gene has similar expression patterns in mouse, human and

other vertebrates (58). WT1 expression is limited in time and location. It is expressed in fetal kidney, fetal undifferentiated gonad and genital ridge. During embryogenesis, the highest levels of expression occur in three situations: differentiation of the metanephric mesenchyme into nephrons, formation of mesothelium from the mesenchyme and production of the sex cords from the mesenchyme of the primitive gonad. During nephrogenesis, the level of WT1 expression decreases with the maturation of the nephron. The expression pattern of the WT1 gene in the developing fetus suggests a key role of the gene in developmental regulation in the kidney and gonad. WT1 is also expressed in certain areas of the spinal cord and brain, and in the body wall muscles (59). To characterize the transcriptional control region of the WT1 gene, genomic clones containing the exon 1 were isolated. DNA sequencing revealed WT1 promoter was GC-rich, while DNAaseI protection assays with Sp1 protein identified different binding sites in the promoter. In cotransfection assays, Sp1 stimulated WT1-dependent transcription three- to fourfold, suggesting a possible role in transcriptional regulation of the WT1 gene. It is unclear yet which transcription factor is responsible for the tissue-specific expression of WT1.

Surprisingly, the WT1, a tumor suppressor gene which is supposed to be inactivated in Wilms tumors, is expressed in most Wilms' tumors and by the same cellular constituents as during normal nephrogenesis (61,62). The level of WT1 expression in Wilms' tumors is highly variable with intralobar and perilobar tumors having significantly different expression levels (63). Though in some cases this difference may specifically result from inactivation of the WT1 gene, it

is more likely to be related to the quantity of each cell type within the tumor since each of them has different levels of WT1 expression. WT1 expression in Wilms' tumors occurs mainly in the blastema, immature tubular structures and the glomeruloid bodies. WT1 mRNA is almost undetectable in the mature tubules or stromal tissue. In addition, the level of WT1 expression has been found to be inversely related to the degree of differentiation in blastemal tumors (62). Combined with the finding that WT1 mRNA is abundant in normal fetal kidney, these observations suggest that WT1 gene is involved in the kidney development and most possibly in differentiation of blastemal components. The WT1 gene product may normally act at an early point in the pathway responsible for the induction of the metanephric blastema either inducing differentiation or inhibiting proliferation. Loss of WT1 function may result in errant differentiation or continued proliferation thus contributing to Wilms' tumor development.

Loss of WT1 function can not explain all of Wilms' tumorigenesis. If one was to postulate that the WT1 gene is the only gene responsible for a specific early step in renal differentiation, it could not explain the variation in differentiated histological components of Wilms' tumor. It is quite possible that the WT1 gene does not control the entire multistep process of nephron formation and that there is another gene(s) involved in the development of Wilms' tumor. This hypothesis is supported by the finding that many Wilms' tumors do not have mutations in the WT1 gene (64).

I.3.1.4. WT1 mutation

The WT1 gene has been found to be mutated in about 10-15% of Wilms' tumors analyzed (65). Deletions within the WT1 gene detected by Southern blotting as well as subtle mutations have been demonstrated in these tumors. These mutations include missense alterations within the WT1 zinc fingers which are thought to disrupt DNA binding activity, or nonsense or frameshift mutations which can produce truncated polypeptides. Most WT1 alterations occur within the zinc finger domains with unknown reason. Patients having other congenital anomalies are more likely to have WT1 mutations.

As discussed earlier, constitutional deletions of the 11p13 region including the WT1 gene are characteristics of the WAGR syndrome. Patients with WT and genitourinary abnormalities have been analyzed and found to have intragenic germline deletions confined to the WT1 gene (66,67). These mutations were predicted to produce truncated WT1 products leading to reduced WT1 levels during embryogenesis and abnormal urogenital development.

Constitutional mutations of WT1 have also been found in patients with Denys-Drash Syndrome (DDS) (68,69). Different from deletions in the WAGR syndrome, WT1 mutations in DDS are mainly point mutations affecting DNA binding domains. Molecular studies conducted in individuals with DDS have found that the most common WT1 lesion was a missense ^{1180}C to T transition within zinc finger 3 converting ^{394}Arg to Trp. This conversion is thought to change the binding motif of the WT1 gene and inhibit the activity of the normal WT1 protein. The exact effect of these WT1 mutations in tumorigenesis is still under investigation.

Since WT1 mutations are found in only a small number of Wilms' tumors, other loci must be involved in WT initiation.

I.3.2. WT2(?) at 11P15

1.3.2.1. Beckwith-Wiedemann syndrome (BWS) and 11p15

The Beckwith-Wiedemann syndrome (BWS) is a Wilms' tumor-associated disease characterized by a number of congenital overgrowth features like umbilical hernia, macroglossia, neonatal hypoglycemia, gigantism and hemihypertrophy. About 5-10% of BWS patients will develop tumors, including Wilms' tumor, adrenocortical carcinoma, rhabdomyosarcoma and hepatoblastoma (12). Most BWS cases are sporadic, however, in several familial cases, a dominant pattern of inheritance with variable expressivity has been noted. Genetic linkage analysis in these families has defined a BWS locus at chromosome 11p15.5 (71,72). In these families, BWS is more likely to be inherited from mothers than fathers. Because BWS patients have an increased risk of having Wilms' tumor, this BWS lesion on 11p15 may be important in a pathway that impacts on but is not specific to Wilms' tumor development. The exact mechanism for this association is unclear.

Molecular biological studies have also drawn attention to the chromosome 11p15 region and suggested it as a potential locus for the second WT gene. Loss of heterozygosity at 11p15 has been detected in about 50% of Wilms tumors (73). One study has shown that LOH at 11p13 in WTs did not necessarily involve WT1 mutation (74), suggesting that WT1 was not the mutation being revealed by LOH 11p. In some cases , LOH was found to involve both 11p13 and 11p15; in other cases, LOH was limited to region 11p15.5 (73,75,76,77). This would imply that both 11p13 and 11p15 harbor tumor suppressor genes, they may act alone or in combination giving rise to Wilms' tumor development. In tumors which showed no mutations of the WT1 gene, LOH on 11p15 was noticed. Therefore, at least in these cases, LOH event on 11p15 might be more important in tumorigenesis.

1.3.2.2. Genomic imprinting

The genetic pattern of BWS is complex. In cytogenetically normal children with BWS, uniparental disomy for chromosome 11 was found. A few of BWS cases had been found to have duplication of 11p15 on one chromosome. In this case, the parental origin of the duplicated region was identified as paternal (78). In sporadic Wilms' tumors with LOH involving only 11p15, tumor-specific preferential loss of maternal alleles has been noted (73,79,80), though not as frequently as noticed in BWS patients. The bias of parental origin suggests that the gene or genes are subject to imprinting. Therefore, parental genomic imprinting may play a role in some of the Wilms' tumors having lesions in the chromosomal region 11p15.

Genomic imprinting is the differential modification of the maternal and paternal genetic contributions to the zygote which results in differential expression of the parental alleles during embryonic and adult development (81). It reflects a functional modification not a mutation or a different allele at a specific locus. Genes which are silent on the paternal alleles are referred to as "paternally imprinted" while those silent on the maternal alleles are "maternally imprinted".

Genomic imprinting of certain genes is conserved in mouse and human. It is reasonable to assume that the imprinting of these genes has beneficial effects for normal development. Meanwhile, a lot of evidence has shown that genomic imprinting is related to many human diseases especially some pediatric disorders. i.e. Prader-Willi and Angelman syndrome (82,83), neuroblastoma (84), rhabdomyosarcoma (85) and osteosarcoma (86).

The molecular basis of genomic imprinting is not clear yet, but allele-specific modification of DNA through methylation is proposed to be a mechanism (87,88).

How imprinting affects human tumorigenesis is unknown, although a "gene dosage model" has been postulated. According to this model, LOH with duplication of the active paternal or maternal allele of a gene may double the dose of this gene. A double dosage of the gene may have a function that is absent from the single-dosed gene which can interfere with the normal balance of apoptosis and differentiation in a cell, thus leading to the clonal expansion of a cell population to form a tumor (89). More work needs to be done to reveal the exact mechanism of genomic imprinting and its effect on human diseases.

I.3.2.3. IGF-2 and H19

Two genes on chromosome 11p15 which are implicated in cell growth regulation, insulin like growth factor type 2 (IGF-2) and H19, are potential candidates for the second WT gene.

IGF-2 is a regulatory peptide which is critical for normal growth and differentiation. Its deregulation may lead to the overgrowth of target tissues. It is implicated in the progression of many human tumors and metastasis via different mechanisms (90,91,92). IGF-2 is maternally imprinted (maternal allele is transcriptionally inactive) during human embryogenesis (93). Increased levels of IGF-2 have been demonstrated in many human tumors, such as Wilms' tumor (94,95), rhabdomyosarcoma (96) and lung cancer (97). The increase may be potentially accomplished by a variety of mechanisms including: loss of imprinting (LOI), loss of heterozygosity (LOH) with paternal duplication and loss of transcriptional suppression. In Wilms' tumor, approximately 70% of the cases undergo relaxation or loss of imprinting of the IGF-2 gene (94) and contain two active copies. Up to now, no direct effect of IGF-2 on Wilms' tumor development has been demonstrated. Since higher levels of IGF-2 expression in human tumors is assumed to be a result of double gene dosage from the

transcriptionally active paternal allele through different mechanisms, IGF-2 is thought to play an oncogenic role in tumorigenesis.

H19, paternally imprinted, has a putative embryonal growth-promoting function and is located just 200 bp from the IGF-2 gene on 11p15.5. The exact biological role of H19 is unknown. It has been found to be expressed in differentiating fetal cells and some human tumors like testicular cancer (98) and bladder cancer (99). Since H19 was found to be expressed preferentially in the advanced stages of human bladder cancer, it was thought to be an onco-developmental marker of bladder tumor progression and to have an oncogenic property in bladder cancer (99). On the other hand, other evidence showed that H19 might in fact be a tumor suppressor gene. Biallelic expression and decreased expression levels due to LOI (loss of active maternal allele) were observed more frequently in some tumors including WT (95,100,101,102,103), suggesting that loss of the active allele was a contributor to tumor development. This hypothesis was tested directly in two embryonal tumor cell lines, RD and G401 (104). The G401 cells were derived from a malignant rhabdoid tumor, had low expression of H19 and became nontumorigenic when chromosome 11p15.5-11p14 was introduced by micro-cell fusion. Cell lines expressing at least 10-fold more H19 mRNA showed a remarkable reduction in colony formation after transformation by H19. The overexpression of H19 in the cell line RD which was derived from an embryonal rhabdomyosarcoma was also shown to suppress tumorigenecity. Consistent with this observation, H19 RNA was undetectable by Northern blotting in two primary Wilms' tumors that had lost the maternal allele. In Wilms' tumors, LOH on 11p15.5 involves preferential loss of the maternal allele. Since H19 is paternally imprinted, LOH at this locus may result in double copies of the imprinted paternal allele and loss of function of H19. This suggests a suppressor property of H19 in Wilms' tumor.

How the epigenetic changes at the H19 and IGF-2 genes contribute to Wilms' tumorigenesis is unknown. How and when the changes occur are still under investigation (105).

A summary of the H19 and IGF-2 imprinted domain on chromosome 11p (106) is shown on Figure 2. As shown, the paternal allele of IGF-2 can stimulate normal cell growth while the active maternal H19 exerts a repressive function. The balance of these two genetic effects can determine normal cell growth. In Wilms' tumors with LOI, the maternal allele reverts to a paternal epigenotype, which breaks the normal balance and causes increased cell growth. This model is supported by the finding that, in some Wilms' tumors, the biallelic expression of IGF-2 is associated with undetectable level of H19 (107). Thus the imprinting of the maternal IGF-2 is relaxed as a result of LOI and the gene is expressed biallelically. Meanwhile, the previously active maternal copy of H19 is imprinted and the gene has two imprinted alleles with decreased expression.

In addition to IGF-2 and H19 on 11p15.5, a cyclin-dependent kinase inhibitor $p57^{KIP2}$ has recently been postulated as a third possible candidate. Cellular proliferation is controlled by a protein complex of cyclins and cyclin-dependent kinases (CDKs). The CDK subunits can phosphorylate cell cycle-regulatory proteins to release cells from cell cycle arrest. To keep normal cell growth, cyclin-dependent kinase inhibitor (CDKI) proteins bind with cyclin-CDK complexes and inactivate their catalytic domains. Mutations in the CDKI proteins are supposed to result in uncontrolled cell proliferation. Therefore, it is reasonable to assume that the CDKI proteins are candidates for tumor suppressor genes. $p57^{KIP2}$ is a CDKI protein. The overexpression of the $p57^{KIP2}$ gene can cause growth arrest of mouse cells, implying a tumor suppressive role of this gene (108). The $p57^{KIP2}$ gene is located on 11p15.5, about 500 kb centromeric to the IGF-2 gene, and seems to have a preferential expression of

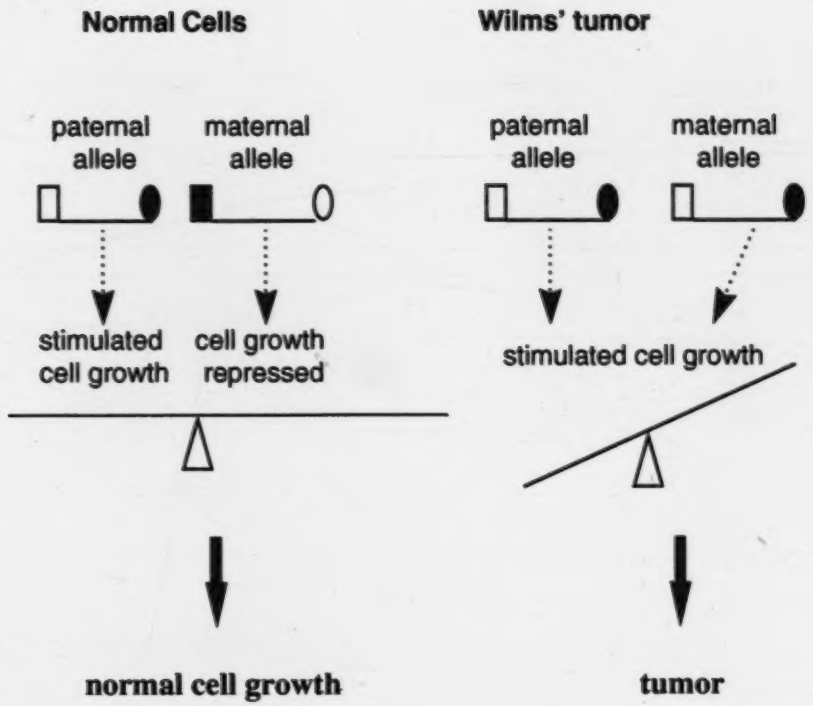


Figure 2. Model for mechanism of LOI in IGF-2 and H19 genes in Wilms' tumors.

active IGF-2 gene

imprinted IGF-2 gene

activeH19 allele

imprinted H19 allele

(A) In normal cells, paternal allele of IGF-2 stimulates cell growth, maternal H19 inhibits cell growth. (B) In Wilms' tumors having LOI, maternal allele reverts to a paternal epigenotype. Two copies of paternal allele of IGF-2 causes increased cell growth.

the maternal allele. It has not been confirmed if this gene is truly imprinted, since the paternal allele is also found to be expressed at low levels in most tissues. In fetal brain and some embryonal tumors, the paternal allele even expresses at levels comparable to its maternal counterpart (109,110). Because of its function, the possible selective expression and chromosomal location of the $p57^{KIP2}$ gene, several studies have been undertaken to search for potential mutations of this gene in tumors, mainly WT-related, to find out its role in tumorigenesis. Deletions of the $p57^{KIP2}$ gene or point mutations in the region encoding its inhibitory domain have not been demonstrated in Wilms' tumors analyzed (111,112). It has yet to be determined whether $p57^{KIP2}$ is involved in Wilms' tumorigenesis.

A recent study of Beckwith-Wiedemann Syndrome (BWS) has found a 4th imprinted gene on 11p15 between $p57^{KIP2}$ and IGF-2, KVLQT1 (113). It is a gene that causes the familial cardiac defect long QT (LQT) syndrome. This gene has been demonstrated to be disrupted by chromosomal rearrangements in BWS patients. Its role in Wilms' tumor also needs to be investigated.

The above evidence suggests that there exists a large imprinted domain of contiguous genes on 11p15. Genes residing in this domain may be abnormally imprinted in tumorigenesis.

I.3.3. Additional Wilms' tumor loci

Though genetic alterations on chromosome 11p contribute to a certain percentage of Wilms' tumors, the development of many WT cases appear to be more complex. More and more evidence suggests that Wilms tumorigenesis is a

multistep process involving various genes, and most possibly, different loci are implicated in the pathogenesis of different WT subtypes.

I.3.3.1. Familial loci

Familial Wilms' tumor is rare, occurring in approximately 1% of all WT cases. Epidemiological study has revealed an autosomal dominant pattern with incomplete penetrance (1). The pathogenesis of familial Wilms' tumors is not clear. No currently identified Wilms' tumor genes have been found to be involved in familial cases. Genetic linkage studies in four families with apparently dominant inheritance of Wilms' tumor but with varying penetrance have excluded the short arm of chromosome 11 (115,116) especially the WT1 gene itself (117) as site of the predisposing mutations. Genomic imprinting is also unlikely to be a mechanism for the tumorigenesis in familial cases (118). Since the number of informative families is small, even though a certain degree of linkage might exist, it is in fact very hard to demonstrate linkage to a specific region of the genome through epidemiological studies.

Molecular biological studies have offered some information on the potential predisposing gene(s). Comparative genomic hybridization has been used to analyze tumor specimens of eight familial cases to identify the consistently lost chromosomal regions (119) and found extensive genetic alterations. The most consistent findings with likely biological relevance were deletions of chromosomes 3 (3q12-q21), 4 (4q21-qter), 9 (9p21-pter) and 20p.

A study of a Canadian family which contains seven known cases of Wilms' tumor in three generations has assigned a FWT1 (familial WT 1) gene to 17q12-q21 (120). Further examination of LOH in sporadic WTs for DNA markers within this interval identified no allele loss, suggesting that this FWT1 gene might not be a tumor suppressor gene for sporadic tumors.

I.3.3.2. p53 gene

p53 is a tumor suppressor gene that is involved in many human tumors. Mutations of p53 have been detected in more than 50% of human cancers (121). The role of p53 in tumorigenesis is not well understood but is believed to affect apoptosis. According to this hypothesis, one function of the normal p53 gene product is to induce damaged cells to undergo apoptosis, thereby, preventing the propagation of transforming mutations. Mutations in the p53 gene may release cells from the normal state of growth inhibition and inactivate a p53-dependent apoptotic process (122,123). p53 mutations are noted in advanced and relapsed diseases in different malignancies arising from various cell types and regarded as a late event in tumor progression.

Though p53 mutations are uncommon in Wilms' tumors (124,125), they have been demonstrated to correlate with the anaplastic Wilms' tumor (126,127,128). As described previously, anaplasia is a potent marker of adverse outcome in Wilms' tumors. The linking of anaplasia to poor prognosis lies in the increased resistance to therapy of tumor cells. Up to now, most p53 mutations in

Wilms' tumors have been reported in the anaplastic subtype and are thought to be a molecular marker for anaplastic Wilms' tumors. How p53 affects Wilms' tumor is unknown. One possibility is that p53 may interact with the WT1 protein. One study (129) has confirmed the presence of a WT1/p53 complex in a kidney-derived, wild-type WT1 and p53 transfected cell line BRK. The functional interaction between WT1 and p53 has been observed only with the wild-type proteins. It is proposed that the interaction between them might affect their ability to transactivate their respective targets: in the absence of p53, WT1 activates the transcription of the early growth response gene 1 site; p53's ability to transactivate its target is enhanced when bound to functional WT1.

I.3.3.3. 16q and other chromosomal loci

LOH studies have been used to identify regions frequently deleted in tumors no matter if the remaining chromosome is duplicated or not. It has been demonstrated to be an effective method of identifying chromosomal regions which might harbor tumor suppressor genes. Therefore, it is notable that LOH for 16q loci was found in 20% of Wilms' tumors (130). This finding suggested not only the involvement of 16q in Wilms' tumor development, but also the existence of a tumor suppressor gene(s) in this chromosomal region. Because allelic loss in this region is also found in other cancers such as ovarian carcinoma (131), sporadic breast cancer (132,133), prostate cancer (134) and hepatocellular carcinoma (135), the putative 16q "WT" gene may actually be a universal

suppressor gene which is involved in the development or progression of various tumors.

LOH on 16q in WT has been found to have prognostic significance since the tumor-specific loss of 16q loci is associated with poor outcome of the affected patients (136). One aim of cancer research is to reveal the nature of tumors and the mechanisms of tumorigenesis. A better understanding of a tumor may lead to the improved treatment of the disease. Therefore, it would be very helpful to identify a gene on 16q and define its relationship to the prognosis of Wilms' tumor, since a new prognostic factor might be helpful in further refining current clinical trials for the treatment of Wilms' tumor.

In addition to mutations on 16q, cytogenetic analyses of short-term cultures of WT have demonstrated multiple genetic alterations including trisomy 1, trisomy 2, trisomy 7 and trisomy 20 (137,138). Combined with the loci discussed above, these observations suggest a strong genetic heterogeneity of Wilms' tumor. Different genetic lesions may contribute independently or reciprocally in the formation of various Wilms' tumor subtypes.

II. Differential display polymerase chain reaction

Numerous genetic alterations have been demonstrated in a variety of human disorders. Accordingly, many techniques have been developed to isolate human genes that are responsible for a specific trait or a disease. The most common procedure for identifying predisposing genes is positional cloning (139).

By definition, positional cloning is a method which can isolate a gene localized to a particular region of a chromosome. Generally, it requires a mutant human gene whose inheritance can be traced in family groups by virtue of the phenotype that the mutation causes. The main steps of positional cloning are: 1. Linking of a disease to a chromosomal location. This is carried out by linkage analysis performed in pedigrees involving many families in combination with the use of multiple polymorphic markers (RFLP analysis). These markers are used to position the gene within a certain region on a human chromosome. 2. Constructing genomic DNA libraries containing the region between two RFLP markers in which the target gene resides. Clones obtained from these libraries are used to form a genetic map of the region. Various strategies such as screening of cDNA libraries and sequencing of the region are used to isolate the expressed sequences in the linked area. 3. Analyzing the isolated sequences for the presence of mutations in corresponding patients. Identification of mutations may indicate that the gene is responsible for the disease.

Though many disease-causing genes have been isolated by this technique (140,141), positional cloning is very laborious and time-consuming. It requires large pedigrees with defined phenotypes and involves many complicated procedures. It can be further complicated if one is analyzing heterogeneous or multigenic diseases.

Another widely used gene isolation method is subtractive hybridization, a powerful way to enrich for differentially expressed sequences prior to cDNA cloning. It can be used to select a gene that is expressed uniquely or

preferentially in one of a pair of closely related cell populations. The main procedure is to synthesize cDNA using mRNA from cell types containing the sequence(s) of interest. Synthesized cDNAs are then hybridized with a large excess of mRNA molecules from a second cell type which does not contain the sequence of interest. cDNA sequences corresponding to differentially expressed mRNAs should remain single-stranded after hybridization. These sequences are separated from double-stranded nucleic acids and cloned into a vector library. cDNA libraries prepared after subtractive hybridization can then be used to clone the corresponding genes. This technique has been demonstrated to be effective in finding genes involved in differentiation or tumorigenesis, etc (142,143,144).Subtractive hybridization, however, is mainly a qualitative method rather than a quantitative one. Besides, it is time-consuming and can only compare two cell types at a time.

It is generally accepted that cancer is a result of the accumulation of the activation of cellular oncogenes and the inactivation of tumor suppressor genes. To identify cancer-related genetic factors, it would be beneficial to have a method which can detect all mRNA species expressed in a cell. Both qualitative and quantitative changes in gene expression should be identified by comparing expression patterns in different cells and both activated and inactivated gene expression should be identified. Differential display polymerase chain reaction (DD-PCR) (145), or differential mRNA display, is such a method that is potentially fulfilling all these needs.

II.1. DD-PCR procedure

DD-PCR is a technique designed to detect differential gene expression in cells at the mRNA level. The development of DD-PCR was inspired by the reverse-transcription PCR (RT-PCR) technique (146) which synthesizes a population of cDNAs from mRNAs in the tissue of interest through reverse transcription. The specific cDNA of interest can be amplified using an additional oligonucleotide known to be specific to the sequence of interest. It can be used to confirm or quantitate the differential expression of any gene whose sequence is known. The advantage over RT-PCR is that DD-PCR can display all mRNA species expressed in a particular cell and thus has the potential to identify a spectrum of known or unknown genes that are differentially regulated in cells. The basis for this technique is that, in principle, every individual mRNA molecule of the estimated 20,000 transcribed genes in a cell can be reverse transcribed and amplified by PCR. The general strategy for DD-PCR includes the reverse transcription of mRNA and amplification of partial cDNA sequences from subsets of mRNAs by PCR (Figure 3).

II.1.1. Reverse transcription

The first strand copy of cDNA is made by using an oligo(dT) primer that has a specific dinucleotide at its 3' end, such as 5'-T_{11}AC. This 3' primer can anneal with the polyadenylated [poly(A)] tail present in most mRNA molecules and thus be anchored to the 3' end of the mRNA. A primer like 5'-T_{11}AC will bind

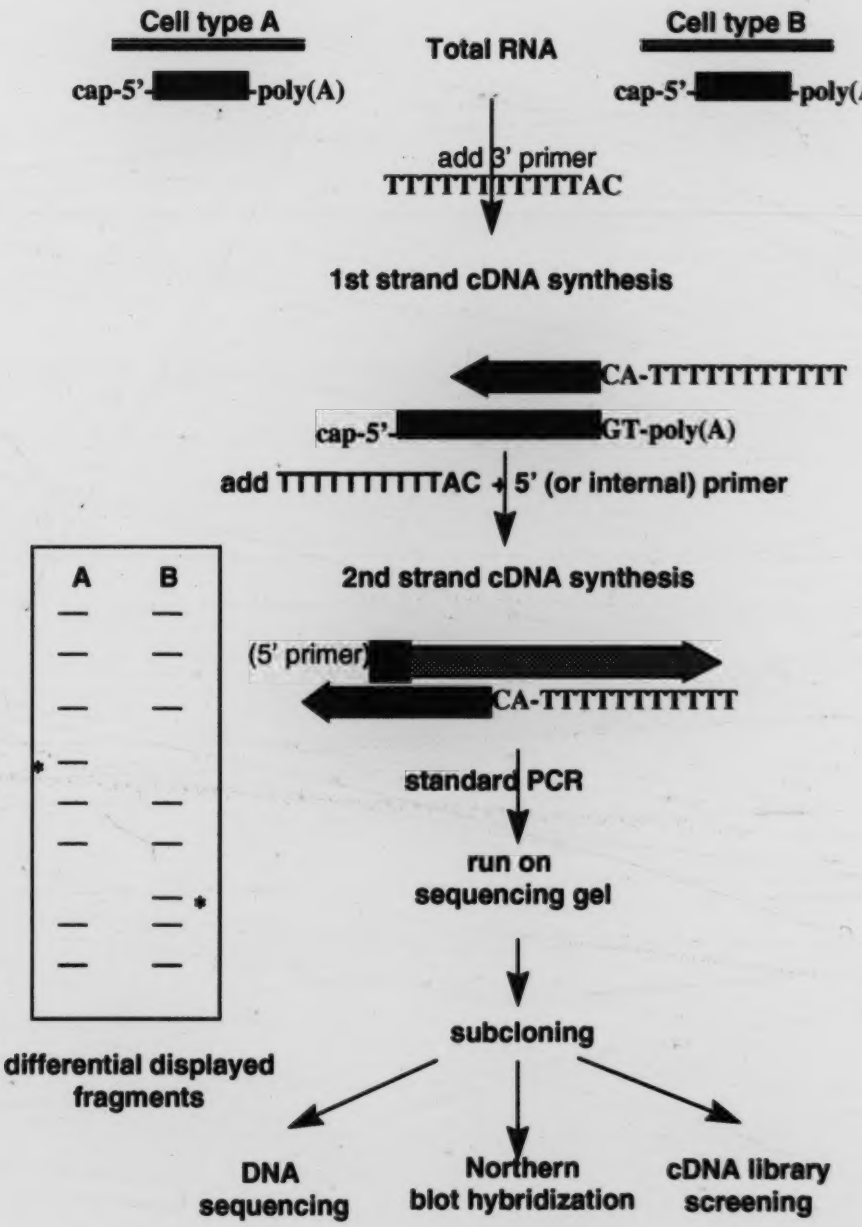
Figure 3. Schematic diagram of regular DD-PCR procedure.

1). Total RNA is reverse transcribed to form the first strand copy of cDNA.

2). With the addition of an arbitrary primer in the reaction, cDNAs to which both primers hybridize are amplified by standard PCR.

3). Differentially displayed cDNAs (marked with asterisk in the figure) are identified by running radio-labeled PCR products on sequencing gel.

4). Northern blot hybridization-confirmed DD-fragments are subcloned and characterized by DNA sequencing or cDNA library screening.


Cell type A
Total RNA
Cell type B
cap-5'-
-poly(A)
cap-5'-
-poly(A)
add 3' primer
TTTTTTTTTTTAC
1st strand cDNA synthesis
CA-TTTTTTTTTTT
cap-5'-
GT-poly(A)
add TTTTTTTTTTAC + 5' (or internal) primer
2nd strand cDNA synthesis
A
B
(5' primer)
CA-TTTTTTTTTTT
standard PCR
run on
sequencing gel
subcloning
differential displayed
fragments
DNA
sequencing
Northern
blot hybridization
cDNA library
screening

mRNA species having GT immediately upstream of the poly(A) tail, and only this subpopulation will be reverse transcribed. By probability, this primer will detect 1/12 of the total mRNA in a cell omitting T as the 5' base in the dinucleotide.

With the addition of a 5' or arbitrary primer, the second strand cDNA can be synthesized. This arbitrary primer does not have to anneal to the 5' end of the mRNA but can bind anywhere 5' to the 3' end [poly(A) tail] in the cDNA strand. The annealing positions will be randomly distributed along different cDNA strands, so cDNAs of different sizes will be generated. cDNA species to which both primers hybridize are then amplified through polymerase chain reaction.

II.1.2. PCR amplification

According to Liang and Pardee's experiment (145), any cDNA in which the arbitrary primer anneals within 2-3 kb of the poly(A) tail can be amplified by PCR. The best result will be obtained if the annealing position is within 500 bp, a size that can be resolved on a sequencing gel.

Cycle parameters are similar to those of a standard PCR procedure, but a lower annealing temperature of 42°C has been found to be optimal for yield and specificity. A radioactive nucleotide is included in the reaction, so the PCR product can be visualized by autoradiography.

Generally, many bands representing different mRNA species are visualized in each lane. Some of them may be present universally in all compared cells while some may appear only in certain samples due to a potential differential expression of the corresponding mRNA molecules. Bands

showing differential patterns between cells of interest are considered as candidates for differentially displayed mRNA species, cut from the gel, reamplified and further characterized.

II.1.3. Characterization of genes after DD-PCR

The most frequently used initial procedure for characterizing DD-PCR fragments is Northern blot hybridization. The purpose of Northern hybridization is to confirm the differential pattern revealed by DD-PCR, so as to eliminate false positive patterns due to PCR artifacts, genomic DNA contamination or other unknown irrelevant differences. Only cDNAs whose reveal differential expression is confirmed by Northern blot hybridization can be regarded as truly representing the differentially expressed mRNA species. Subcloning of these candidate fragments into an appropriate vector allows further characterization by ensuring an adequate source of cDNA. cDNA sequencing may reveal the identity of the sequence or identify it as a novel gene. To obtain full length clones, it is necessary to screen a cDNA library.

II.2. Application of DD-PCR

Ever since its introduction in 1992 by Drs. Liang and Pardee, DD-PCR has been used widely as an effective way to identify and isolate genes that are differentially expressed among compared cells.

DD-PCR has been used in developmental biology to clone genes differentially expressed in the prenatal and neonatal mammalian brain (147,148),

and in preimplantation embryos (149). Other applications have led to the identification of molecular factors involved in the pathogenesis of varied diseases (150,151). DD-PCR has been mostly utilized in human cancer research attempting to identify and isolate new tumor-related genes (152,153,154,155).

The advantages of this technique lie in its technical ease, the minimal quantities of total RNA required for analysis and its capability to simultaneously identify both upregulated and downregulated genes. Though there are limitations and practical problems, DD-PCR has been demonstrated to be a powerful and effective technique in identifying differentially expressed genes.

III. Objectives of this study

The treatment of Wilms' tumor has advanced dramatically with the development of chemotherapy in the 1960s and made it one of the most successfully treated human malignancies. Currently, most early stage tumors are treated by surgical resection of the affected kidney and dissection of any other sites involved with tumor cells such as lymph nodes. This is followed by chemotherapy, with radiation therapy reserved for tumors with adverse prognostic indicators. Current survival rates for patients with tumors having favorable histology is more than 85% (22), a rate too high to be increased solely by conducting additional therapeutic trials. For the remaining 15% of patients, the treatment could still be improved if this group could be identified by additional prognostic factors. In this case, intensified therapies could be used for patients destined to relapse while for those with an expected favorable outcome,

it might be possible to decrease the intensity and duration of treatment. Therefore, identifying new genetic prognostic elements or understanding the molecular pathogenesis could benefit the treatment of Wilms' tumor.

Previous LOH studies conducted in our lab revealed a tumor-specific loss of chromosome 16q and an adverse prognostic significance of this loss, suggesting that this region may harbor a new WT gene(s). Considering the molecular biological and clinical significance of the putative 16q gene(s), we undertook DD-PCR analysis of Wilms' tumors trying to identify and isolate this candidate WT gene, genes whose expression was secondarily altered, or novel tumor or outcome-related genes. Since 16q region identified by LOH was very large, it was not feasible to use positional cloning to clone a candidate gene within this region. Therefore, the objective of this study was to use DD-PCR to identify putative tumor-related gene(s), preferably those responsible for the prognosis of the tumor.

CHAPTER II MATERIALS AND METHODS

I. RNA isolation

Seven Wilms' tumor tissue samples of six Wilms' tumor patients obtained from the Pediatric Oncology Group were used in this study. Clinical details and genotypes at chromosomes 1p, 11p and 16q are shown in Table 1. These tumor tissues had been aliquoted and stored at -80°C. For each tumor, total RNA was extracted from several tissue aliquots in TRIzol™ Reagent (GIBCO BRL) according to the manufacturer's direction. RNA pellets were dissolved in RNase-free ddH_2O, stored at -80°C and used for reverse transcription. Generally, 1000-1200 mg of the tissue yielded about 3-4 mg of total RNA.

Poly(A)+ RNA was prepared using Oligo(dT) cellulose columns (Collaborative Biomedical Products). Poly(A)+ RNA pellets were resuspended in RNase-free ddH_2O and stored at -80°C. The yield of poly(A)+ RNA was about 3-5% of the total RNA.

II. DD-PCR

Reverse transcription

For each tumor, 2 µg of total RNA which was heatshocked at 65°C for 2 minutes was reverse transcribed by mixing with reagents such as 5×FSB (first strand buffer), 0.5 mM dGATC, 10 U/µl RNase inhibitor, 10 mM DTT, 10 mM spermidine, 200 U/µl reverse transcriptase and 0.67 µg/µl external primer.

Table 1. Information of the patients and the tumors used in the study.

		clinical features			genotype by chromosomal location				
patient No.	tumor No.	tumor stage	histology	relapse status	1p	11p13	WT1	11p15	16q
1	E97T	III	FH	-	R	R	R	R	R
2	E98T	III	FH	-	R	R	R	R	R
3	E108T	III	FH	-	R	R	R	R	R
4	E53T	II	FH	+	R	R	R	R	L
5	E171TR	III	FH	+	R	R	R	R	L
5	E171TL	III	FH	+	R	R	R	R	L
6	E301T	NA	ANA	+	R	R	NA	R	L

Notes:

1) Patient No. 5 had bilateral tumors, with tumor E171TR excised from the right kidney and E171TL from the left.
2) Tumor stages are defined as (from NWTS 3): stage I: tumors limited to the kidney; stage II: tumor extension beyond the kidney margins with complete excision; stage III: lymph node involvement or incomplete total surgical resection; stage IV: hematogenous metastases to distant sites; stage V: bilateral renal involvement; NA, not available.
3) FH, favorable histology; ANA, anaplastic histology.
4) Relapse status: -, tumor did not recur in the patient; +, tumor recurred.
5) R, heterozygosity retained in tumor; L, loss of heterozygosity detected in tumor.

The reverse transcription reaction was carried out at 42°C for one hour. The final reaction product was either stored at -80°C or used immediately for PCR amplification. All reagents except primers were purchased from GIBCO BRL.

PCR amplification

PCR reactions were performed with each of twenty internal (5') primers in combination with each of two external (3') primers (Table 2). For each reaction, 2 µl of cDNA was amplified by a standard PCR procedure with a radioactive nucleotide, α-^{35}S-dATP (1200 Ci/mmol), in the reaction. 2 U/µl Taq DNA polymerase (Pharmacia Biotech) was added at the beginning of the reaction. The total of 20 µl reaction mixture was amplified through 40 cycles of 94°C - 30 sec / 41°C - 90 sec / 72°C - 30 sec. At the end, 7.3 µl sample loading buffer was added to stop the reaction. PCR products were either stored at -80°C or run on a 6% sequencing gel immediately. Each PCR product was loaded on two different sets of lanes using 5 µl for each loading, the second set separated from the first by two hours to ensure that large as well as small fragments could be detected. cDNA fragments were electrophoresed at 70-80 W. The sequencing gel was vacuum dried at 80°C for 1 hour and exposed to Kodak X-ray film at room temperature for 1 or 2 days.

Reamplification and purification

Bands of interest were excised and eluted in ddH_2O. cDNAs were precipitated at -80°C with 3 M NaOAc (pH 7.0), 20 mg/ml glycogen, ethanol and

Table 2. Sequences of the primers used for reverse transcription, PCR and DNA sequencing.

primer		sequences
external	RED-C	5' TTTTTTTTTT (CAG) C 3'
	RED-A	5' TTTTTTTTTT (CAG) A 3'
internal	OPB-01	5' GTTTCGCTCC 3'
	OPB-02	5' TGATCCCTGG 3'
	OPB-03	5' CATCCCCCTG 3'
	OPB-04	5' GGACTGGAGT 3'
	OPB-05	5' TGCGCCCTTC 3'
	OPB-06	5' TGCTCTGCCC 3'
	OPB-07	5' GGTGACGCAG 3'
	OPB-08	5' GTCCACACGG 3'
	OPB-09	5' TGGGGGACTC 3'
	OPB-10	5' CTGCTGGGAC 3'
	OPB-11	5' GTAGACCCGT 3'
	OPB-12	5' CCTTGACGCA 3'
	OPB-13	5' TTCCCCCGCT 3'
	OPB-14	5' TCCGCTCTGG 3'
	OPB-15	5' GGAGGGTGTT 3'
	OPB-16	5' TTTGCCCGGA 3'
	OPB-17	5' AGGGAACGAG 3'
	OPB-18	5' CCACAGCAGT 3'
	OPB-19	5' ACCCCCGAAG 3'
	OPB-20	5' GGACCCTTAC 3'
sequencing	Forward	5'-d[GTAAAACGACGGCCAGT] 3'
	Reverse	5'-d[CAGGAAACAGCTATGAC] 3'

resuspended in ddH_2O. Eluted cDNAs were then reamplified by polymerase chain reaction using the same set of primers and reaction conditions as used for the original differential display with the individual reaction reagents and the final volume being doubled. For each reaction, 30-40 µl of reamplified product with the sample loading buffer added was run on a 5% polyacrylamide gel under 100V for 2-3 hours. A molecular weight marker was used to estimate sizes of the fragments. cDNA band which was visualized by EtBr staining were excised from the gel and recovered.

III. Molecular cloning

A TA® Cloning Kit from Invitrogen was used to subclone the cDNAs of interest into a pBluescript vector, pCR™2.1. Fresh PCR product (less than one day old) was ligated into the vector by using reagents offered in the cloning kit. The ligation reaction mix was incubated in 16°C waterbath overnight. If not used for transformation immediately, it was stored at -20°C. Recombinant plasmids were transformed into Invα'F cells using procedures provided by the manufacturer. Transformed cells were spread on LB plates, incubated at 37°C for at least 18 hours and shifted to 4°C for 2-3 hours to permit proper color development. White or white-centered colonies which were supposed to contain recombinant plasmids were picked out and grown in 10 ml LB medium at 37°C overnight. Plasmid DNAs were prepared by standard methods described previously (156).

To further characterize the recombinant plasmids, restriction enzyme digested plasmid DNA was run on both a 1% agarose gel and an 8% polyacrylamide gel. A Southern blot containing DNA fragments of all recombinant plasmids from a given transformation was prepared using standard alkaline transfer after agarose gel electrophoresis (156). Polyacrylamide gel electrophoresis was used to identify sizes of the inserts and to pick out an insert which was present in most recombinants to be used as a probe for the Southerns. This "probe" DNA was labeled by α-^{32}P-dCTP (3000 Ci/mmol) with the aid of Klenow enzyme (Pharmacia Biotech) by using a random primed labeling procedure. The labeled probe was purified using a NucTrap® probe purification column (Stratagene) to eliminate unlabeled nucleic acids. Specific activity of the radiolabeled DNA was measured to check the labeling efficiency. A reading with 10^9 cpm/µg DNA was regarded as a mark of successful labeling. The probe was then hybridized to the Southern blot prepared previously. The hybridization procedure included prehybridization in 0.1×SSC and 0.5% SDS at 65°C for 10 minutes and hybridization in 50% formamide / 0.12 M $NaHPO_4$ (pH 7.2) / 0.25 `M NaCl / 7% SDS at 42°C overnight. After hybridization, the blot was exposed to X-ray film (Kodak) at -80°C for 1-2 hours.

IV. Northern hybridization

2 µg of polyA+ RNA from each of the seven tumors used for the original differential display was separated on a 1% agarose gel (containing 10×MOPs and formaldehyde) and transferred to Nitroplus nitrocellulose transfer membrane

(Micron Separations Inc.). The Northern blot was baked at 80°C under vacuum for 2 hours and stored at 4°C for hybridization.

Northern blot was prehybridized for 4 hours and hybridized overnight to a "probe" DNA which was labeled by α-^{32}P-dCTP (3000Ci/mmol) at 42°C in a solution containing 50% formamide, 20×SSC (25%), 100×Denhardts' (5%), 1M NaH_2PO_4 (pH 6.5) and salmon sperm DNA solution (15%). The hybridized blot was exposed to X-ray film at -80°C for 4 hrs to 1-2 days.

V. DNA sequencing

T_7 sequencing Kits and reverse primers were purchased from Phamacia. Procedures were recommended by the manufacturer.

Sequencing products were electrophoresed under 65W on a 6% denaturing polyacrylamide gel by running three sets of loading through different periods of time (8 hours, 5 hours and 2 hours). 5µl of the sequencing mixture was used for each loading. The sequencing gel was dried at 80°C under vacuum for 1 hour and exposed to X-ray film at room temperature for 1-2 days.

VI. Chromosome localization

Murine or hamster/human hybrid cell lines, each containing one individual human chromosome, were obtained from ATCC and grown in Dr. McDermid's, Dr. Godbout's and our labs. 10µg of each human chromosome specific hybrid (chromosome 1 to chromosome 22 and X, Y chromosomes), human, hamster and murine control DNAs were digested with EcoRI, separated on 1% agarose

gels and transferred to the Hybond N+ nylon transfer membrane. These Southern blots were hybridized with p^{32}-radio-labeled plasmid inserts using the Southern hybridization procedure mentioned earlier.

CHAPTER III RESULTS

I. DD-PCR

Seven tumors from six Wilms' tumor patients obtained from the Pediatric Oncology Group were used for this study (Table 1 on page 36). Patient No. 5 had bilateral tumors, tumor E171TR was excised from the right kidney while E171TL was from the left side. All tumors were pathologically classified as Wilms' tumors stages II to III and all were of favorable histology except for E301T which was of anaplastic histology. The patients had no associated congenital anomalies except for patient No.2. No patients had family histories of Wilms tumor. These seven tumors were divided into two categories by their genotypes on chromosomes 1p, 11p and 16q as well as by their relapse status. Genotype information was obtained from previous RFLP and PCR-based polymorphism analysis of these tumors in our laboratory. The polymorphic DNA markers used were: D1Z2 (1p36) and D1S7 (1p33-35) on the short arm of chromosome 1; D11S16, FSH, WT1 and D11S914 in the 11p13 region; HRAS1, INS and D11S922 for the region of 11p15; D16S7 (16q24), CTRB (16q24.1) and HP (16q22) on the long arm of chromosome 16. Tumors E97T, E98T and E108T had no LOH detected by these polymorphic markers and had not recurred and were therefore grouped as category I samples. Tumors E53T, E171TR, E171TL and E301T belonged to category II since they had no LOH on 1p and 11p (p13 and p15), but they had LOH on chromosome 16q and tumors were known to have recurred.

The quality of total RNA extracted from several tumor aliquots for each tumor was tested by running on an agarose gel. After staining with ethidium bromide, the 28S rRNA band was nearly twice as bright as the 18S rRNA, suggesting little degradation of the RNA samples. PolyA+ RNA was prepared from total RNA by passing through oligo(dT) columns and stored at -80°C.

Thirty DD-PCR reactions were performed with the combination of two external primers, RED-C paired with twenty internal "OPB" primers and RED-A combined with first 10 OPB primers listed in Table 2. ^{35}S-labeled PCR products were run on 6% denaturing polyacrylamide gels. Most primer combinations worked for every tumor, that is, every tumor lane had PCR products (bands). For two or three primer sets, there were one or two blank lanes on the gel, suggesting a failure of amplification of cDNAs in the tumors. The reasons for this was unknown. Tumors which failed to amplify did so with other primer sets, so the failure of amplification was likely due to the quality of RNAs. Approximately 50 bands could be detected in each sample for each set of primers (Figure 4). Most bands were present in all tumor samples with only a small portion of bands absent from some lanes. Bands of seemingly the same size were assumed to represent the same specific mRNA species. A fragment was considered to have a differential display pattern if it did not occur in all or most tumor samples of one category but was present in most or all of the other. Not all the differentially displayed fragments were regarded as candidates for this study since many factors might have contributed to the differential pattern in addition to LOH 16q and relapse-related factors. Only those fragments showing a consistent

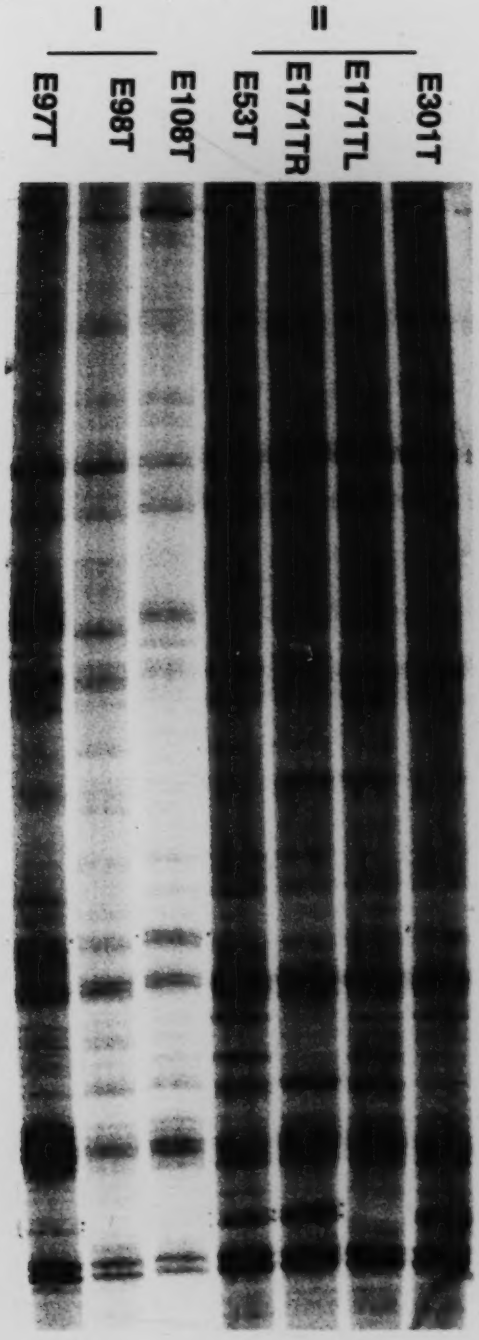


Figure 4. A typical DD-PCR gel (DD-PCR products run on a sequencing gel).

PCR products were run on a 6% denaturing polyacrylamide gel. Usually, approximately 50 bands could be displayed in each sample lane. Tumors E97T, E98T and E108T belonged to Category I which had no LOH on chromosomes 1p, 11p and 16q, and the tumors did not recur. E53T, E171TR, E171TL and E301T were Category II tumors, since they had no LOH on 1p and 11p, but lost 16q and the tumors recurred in patients. Fragment marked with * had absence in some tumors, but it was not a consistent pattern, therefore, the fragment was not a candidate. Fragment marked with arrow had a consistent differential display pattern (was absent completely in Category I and present in 3/4 Category II tumors) and was chosen as candidate.

difference between the two categories of tumors were chosen as candidates. As shown in Figure 4, the fragment marked with asterisk appeared in one of category I tumor, E97T, and two of category II tumors, E53T and E171TL. Though it had a differentially expressed pattern, it was not significantly different between the two categories. Therefore, it was not selected as a candidate DD-fragment. The fragment marked with arrow, though not having a perfect differential pattern between the two categories, was absent from nearly all category I tumors and present in most (3/4) category II samples. Fragments like this were chosen as candidates.

Twenty-three candidate DD-fragments were identified by DD-PCR screening (Table 3). Most of their bands were faint compared to non-candidate bands (data not shown). Nearly half of these fragments, fragment 2 for example, were present in most or all category II lanes with absence from most or all category I tumors, implying that the corresponding genes might act like oncogenes. This display pattern was therefore called the "activated or oncogene" pattern. On the contrary, "repressed or tumor suppressor gene" patterns referred to those fragments having bands present in all or most category I tumors which were absent from most or all category II tumors. Genes corresponding to this set of fragments such as fragment 4 (Table 3) might have tumor suppressor functions and the loss of 16q might result in the loss of gene function.

Table 3. Twenty-three differentially displayed fragments from DD-PCR.

Fragment No.	DD-PCR No.	Primer set RED / OPB	Category I			Category II			
			E97T	E98T	E108 T	E53T	E171TR	E171TL	E301T
1	001'	C/01	+	-	-	+	+	?	+
2	006	C/06	-	-	-	+	+	+	+
3	006	C/06	+	-	-	+	+	+	+
4	006	C/06	+	+	+	-	-	+	-
5	007	C/07	+	+	+	+	-	-	-
6	007	C/07	+	+	+	+	-	-	-
7	007	C/07	-	-	-	+	+	+	+
8	007	C/07	-	-	-	+	+	+	+
9	008	C/08	-	-	-	+	+	+	+
10	008	C/08	+	+	+	-	-	-	-
11	009	C/09	-	-	-	+	+	+	+
12	011	C/11	+	+	+	+	-	-	?
13	011	C/11	+	+	+	+	-	-	+
14	014	C/14	-	-	-	+	+	-	+
15	014	C/14	-	-	+	+	+	+	+
16	016	C/16	+	+	+	+	-	-	-
17	012	C/12	-	+	+	-	-	-	-
18	012	C/12	+	+	+	-	-	-	-
19	022	A/02	-	-	-	+	+	+	+
20	025	A/05	-	-	-	+	+	+	+
21	025	A/05	-	-	-	-	+	+	+
22	025	A/05	-	-	-	+	+	+	+
23	026	A/06	-	-	-	+	+	+	+

Notes:

1) Category I tumors did not relapse and had no LOH on 1p, 11p and 16q.
2) Category II tumors had no LOH on 1p and 11p, but had LOH for 16q and tumors recurred in the patients
3) +, band present; -, band was absent; ?, uncertain signal

To get enough DNA for further analysis, each band of these 23 DD-fragments was excised from the sequencing gel, eluted and prepared for PCR reamplification.

Eleven fragments which had the most consistent differential patterns were reamplified using the same set of primers and cycling conditions as for the original DD-PCR (Table 4). With the use of a DNA size marker during electrophoresis of the reamplified PCR products, the length of the original DD-fragment could be estimated. These 11 fragments ranged in size from 160 bp to 620 bp as listed in Table 4. Most of them were shorter than 500 bp. Following reamplification, 9 out of 11 fragments (except fragments 7 and 8) yielded single-sized reamplified products, excluding the possibility of contamination by adjacent bands during excision. Both fragments 7 and 8 showed a perfect "activated or oncogene" pattern by DD-PCR (Figure 5(A)) since they had bands in all category II tumors but were completely absent from category I. Their reamplified PCR products each contained multiple bands, though a major reamplified band with a size of 190 bp for fragment 7 and 160 bp for fragment 8 could be distinguished on the gel (Figure 5(B)). These major reamplified bands of fragments 7 and 8 were present consistently in each of the category II tumor PCR reamplification and so were assumed to represent the desired DD-fragments seen on the DD-PCR gel. The presence of other cDNA fragments may affect the ligation of the desired fragment into the vector, making it difficult to identify the target recombinants later. Therefore, purification of the desired reamplified cDNAs was necessary and performed before subcloning.

Table 4. Eleven reamplified and further characterized DD-fragments.

Fragment No.	Size (bp)	DD-PCR	Reamplification	Northern confirmation	Sequencing & Chromosomal location
2	280	activated pattern	two bands for each fragment in each sample	no signal	human eIF 5 mRNA
*4	180	repressed pattern	single reamplified product	smear	human AP-3 complex delta subunit mRNA
7	190	activated pattern	multiple bands	-	-
8	160	activated pattern	multiple bands	-	-
9	310	activated pattern	single reamplified product	no signal	human mitochondrion (5')
*10	260	repressed pattern	single reamplified product	not confirmed	no known human genes
11	340	activated patter	single reamplified product	no signal	no known human genes
*16	280	repressed pattern	single reamplified product	no signal	no known human genes
*18	210	repressed pattern	single reamplified product	partly confirmed	no known human genes; locates on chromosome 3
19	280	activated pattern	single reamplified product	not confirmed	no known human genes
20	620	activated pattern	single reamplified product	no signal	human mitochondrion (3')

Notes:

1) "Activated" pattern, bands of a fragment mostly presented in category II tumors; "repressed" pattern, bands of a fragment were absent from most or all category II samples.
2) Category I tumors, no loss on chromosomes 1p, 11p and 16q with no relapse. Category II tumors, no loss on chromosomes 1p and 11p, with LOH on 16q; tumor recurred

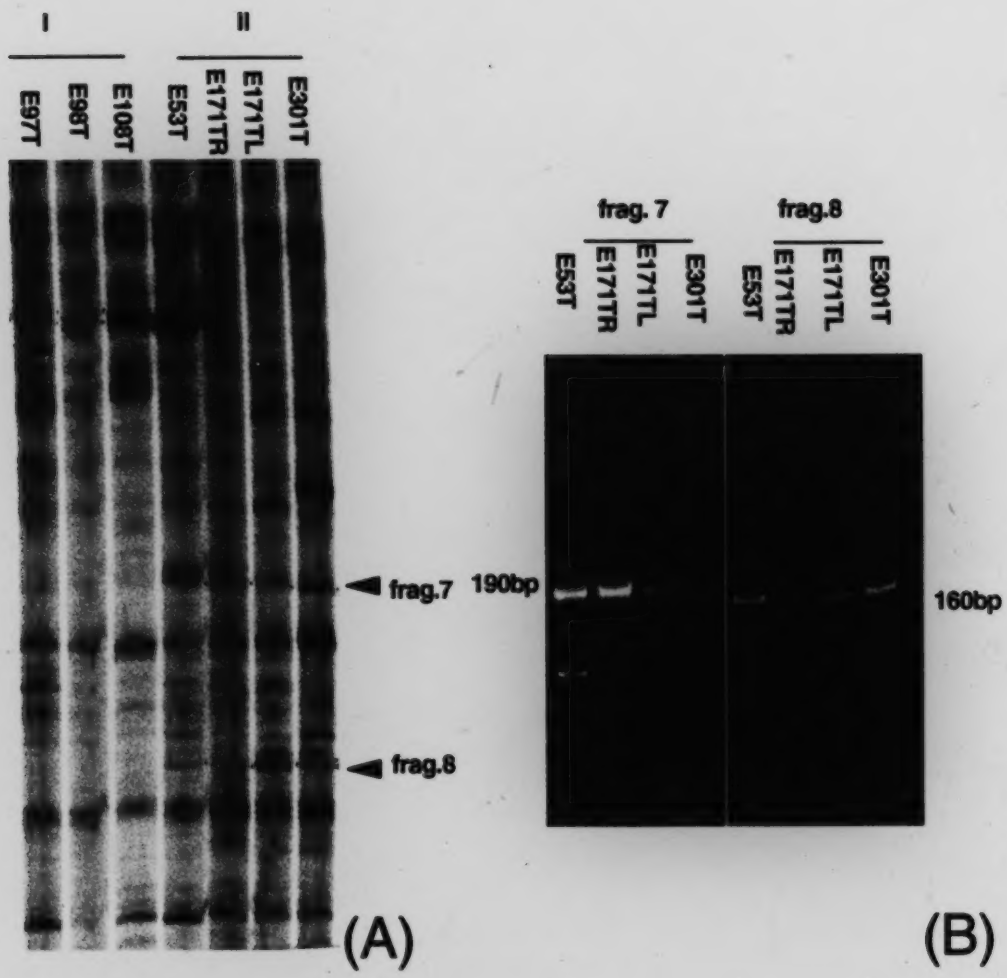


Figure 5. Reamplification of Fragments 7 and 8.

(A). DD-PCR result of fragments 7 and 8. Category I tumors were those having no LOH on chromosomes 1p, 11p and 16q. They did not recur in the patients. Category II tumors had no LOH on 1p and 11p, but lost 16q. The tumors recurred in patients. Both fragments were present in an "activated oncogene" pattern, that is, they were absent from all Category I.

(B). Reamplified fragments 7 and 8 contained multiple bands. Seen from the gel, bands with the sizes of 190 bp for frag.7 and 160 bp for frag.8 had the strongest density, and were selected as the desired fragments representing the original DD-fragments 7 and 8. Other bands may have resulted from contamination or artifact of PCR.

II. Subcloning

A TA®Cloning kit from Invitrogen was used to subclone the 11 candidate fragments into a pBlueScript vector pCR™2.1 (Figure 6). This vector was modified at the unique EcoRI site during preparation. Therefore, the inserts (DD-fragments) could be easily recovered by EcoRI digestion after subcloning. The linearized vector has a T overhang at both ends and is supposed to anneal to the A overhang of candidate cDNAs which is added by Taq polymerase during PCR. Since the single 3' A-overhang is often lost if the PCR product is older than one day, Taq polymerase mediated fresh PCR amplified products of the candidate fragments were used for ligation. These 11 fragments were transformed into the competent Inv'αF cells after ligation. A short-term culture for each ligation/transformation reaction was spread on an LB-agar plate containing antibiotic and X-Gal, and incubated at 37°C for about 18 hours. About 30-40 colonies were observed on each plate although only about 10-20% were white, blue-centered or light blue indicating successful ligation/transformation.

To check if the ligation/transformation was successful, 5 to 8 colonies on each plate were picked and grown in LB medium. Isolated plasmids were digested by EcoRI and separated by polyacrylamide gel and agarose gel electrophoresis. Colonies containing inserts which had a similar size as that of the original DD-fragments were considered as candidate clones (Figure 7). As shown in Figure 7, 14/19 of the recombinant plasmids of fragment 19 had an insert with a size of about 280 bp which was similar to the original DD-fragment

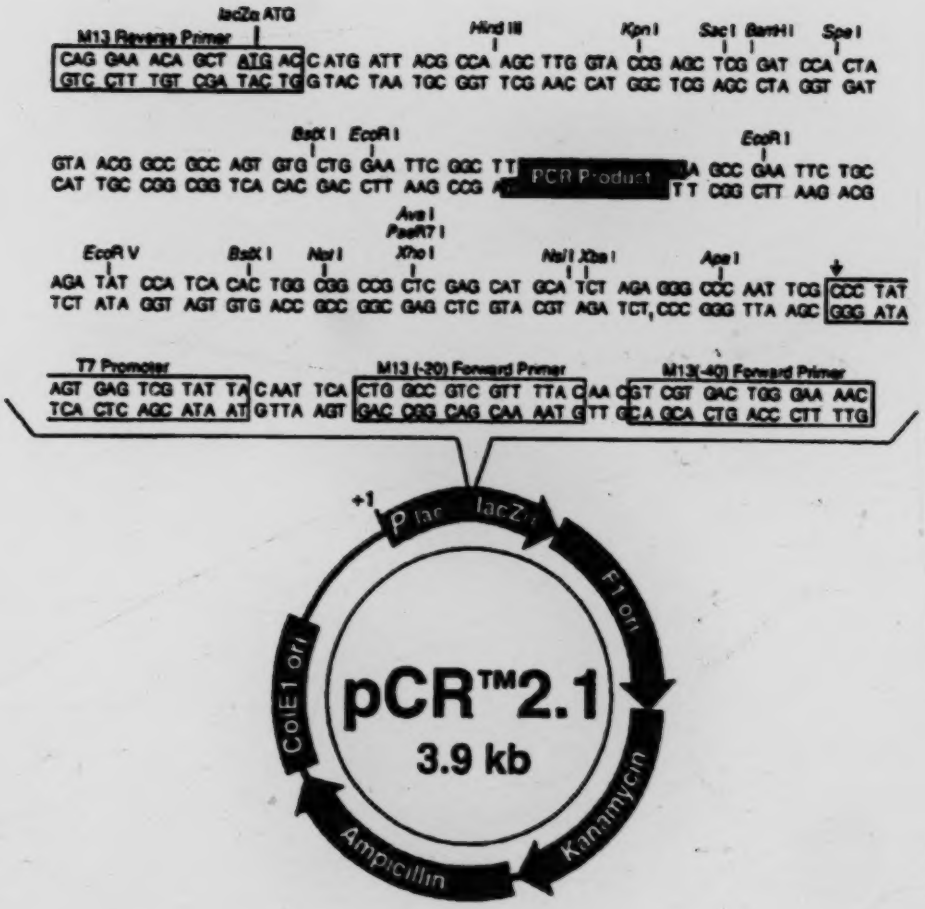

lacZα ATG
M13 Reverse Primer
Hind III
Kpn I
Sac I
BamH I
Spe I
CAG GAA ACA GCT ATG AC C ATG ATT ACG CCA AGC TTG GTA CCG AGC TCG GAT CCA CTA
GTC CTT TGT CGA TAC TG G TAC TAA TGC GGT TCG AAC CAT GGC TCG AGC CTA GGT GAT
BstX I
EcoR I
EcoR I
GTA ACG GCC GCC AGT GTG CTG GAA TTC GGC TT
CAT TGC CGG CGG TCA CAC GAC CTT AAG CCG A
PCR Product
A GCC GAA TTC TGC
TT CGG CTT AAG ACG
Ava I
PaeR7 I
EcoR V
BstX I
Not I
Xho I
Nsi I
Xba I
Apa I
AGA TAT CCA TCA CAC TGG CGG CCG CTC GAG CAT GCA TCT AGA GGG CCC AAT TCG CCC TAT
TCT ATA GGT AGT GTG ACC GCC GGC GAG CTC GTA CGT AGA TCT CCC GGG TTA AGC GGG ATA
T7 Promoter
M13 (-20) Forward Primer
M13(-40) Forward Primer
AGT GAG TCG TAT TA C AAT TCA CTG GCC GTC GTT TTA C AA CGT CGT GAC TGG GAA AAC
TCA CTC AGC ATA AT G TTA AGT GAC CGG CAG CAA AAT G TT GCA GCA CTG ACC CTT TTG
+1
P lac
lacZ
F1 ori
ColE1 ori
pCR™2.1
3.9 kb
Ampicillin
Kanamycin

Figure 6. Map of the linearized vector pCR2.1. (Adapted from Invitrogen's manual). The vector is modified at the unique EcoRI site during preparation. The size of the vector is about 3.9 kb. PCR product can be inserted directly into the vector, thus flanked on each side by EcoRI sites. The sequencing of the fragments took the advantage of the presence of M13 reverse and forward primer sites.

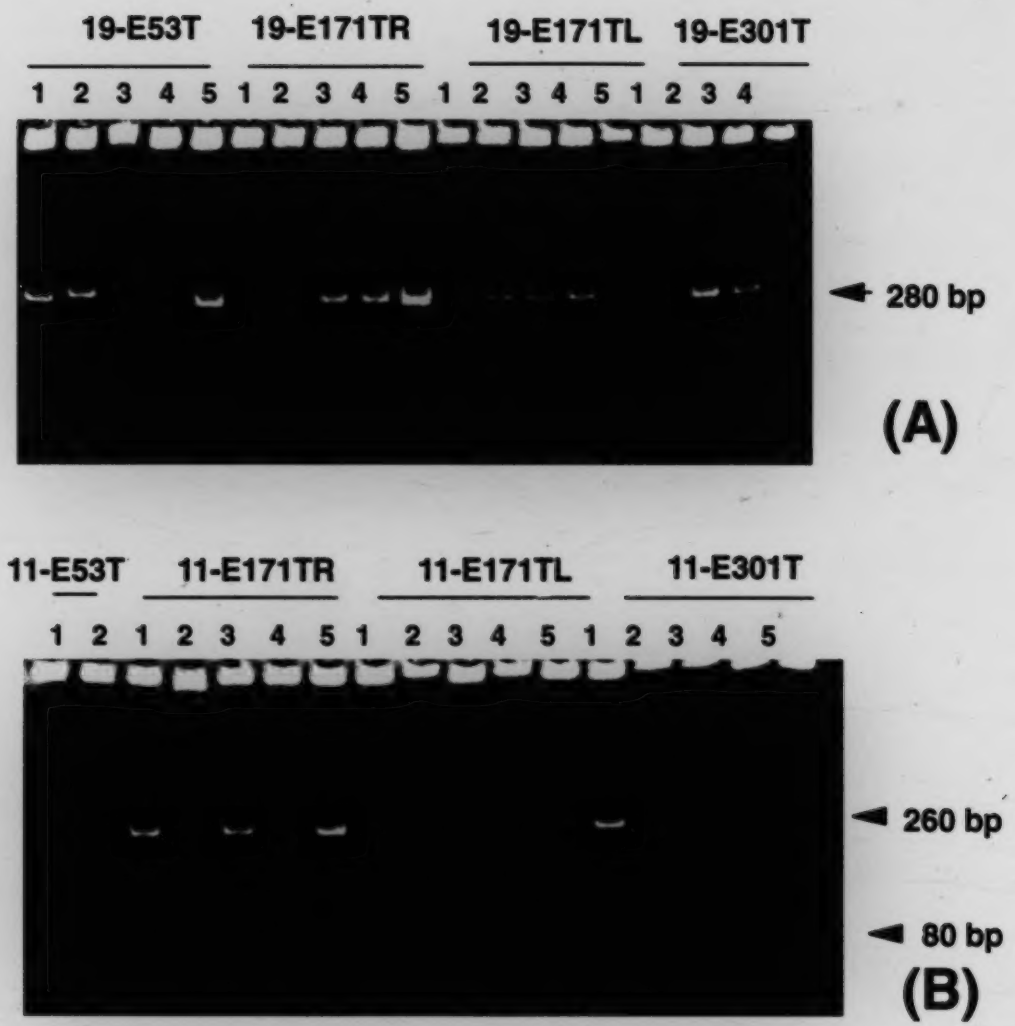


Figure 7. EcoRI digested plasmids.

(A). Plasmids with inserts from Fragment 19. 14 /19 of plasmids had inserts with similar size, 280 bp, indicating that they likely contain the same or the original DD-Fragment 19. There is no EcoRI site within the fragment, so only one fragment was obtained in addition to the linearized vector for these sample.

(B). Plasmids of Fragment 11. 5 / 17 of samples contain bands with similar pattern and sizes, two bands of 260 bp and 80 bp. The original fragment 11 is 340 bp long. suggesting that one EcoRI site exists in the sequence.

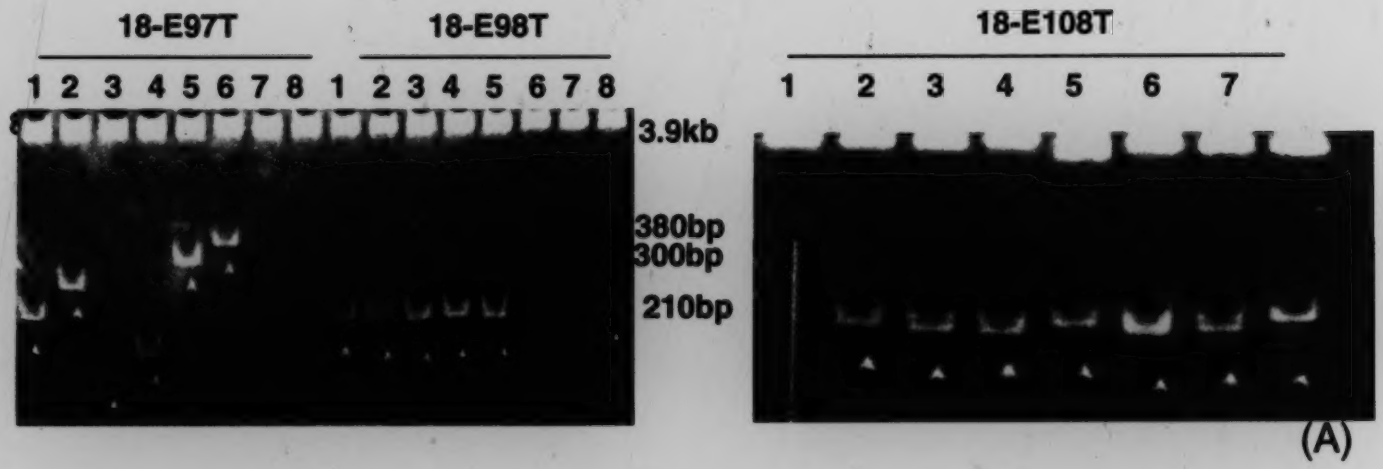
19. Some of the remaining plasmids such as 19-E53T-3 had no insert or some like 19-E53T-4 had an aberrant size DNA. The pattern of a single band following EcoRI digestion suggested that no EcoRI site existed within the insert (Figure 7(A)). If one or more EcoRI sites existed, there should be more than one band in addition to the vector in each plasmid lane. As shown in Figure 7(B), the original DD-fragment 11 was 340 bp. Two inserts of 260 bp and 80 bp were present after EcoRI digestion, implying the existence of an EcoRI site within the fragment.

For each DD-fragment, one Southern blot containing EcoRI digested plasmids from all selected colonies was hybridized with an insert recovered from one of the candidate recombinants. The purpose was to confirm that the candidate colonies contained not only similar-sized but exactly the same insert. For example, after polyacrylamide gel electrophoresis (Figure 8(A)), an insert with a size of 210 bp was present in 14 out of 24 plasmids which were supposed to contain fragment 18. Since the original DD-fragment 18 was estimated to be about 210 bp long, inserts of this size were assumed to represent the original candidate DD-fragment 18. To ensure that they had the same DNA sequence, a Southern blot was prepared with 21 (out of 24) plasmids and hybridized with one of the inserts 18-E108T-6 (Figure 8(B)). Plasmids from the seventh and eighth colonies (marked 18-E97T-7and 18-E97T-8 in Figure 8(A)) were not included in the blot because they contained no inserts. In general, Southern hybridization gave consistent results to that of the polyacrylamide gel electrophoresis. 18-E97T-1 had a 210 bp insert which was similar to that of 18-E108T-6. Southern hybridization confirmed that their two inserts were the same since 18-E108T-6

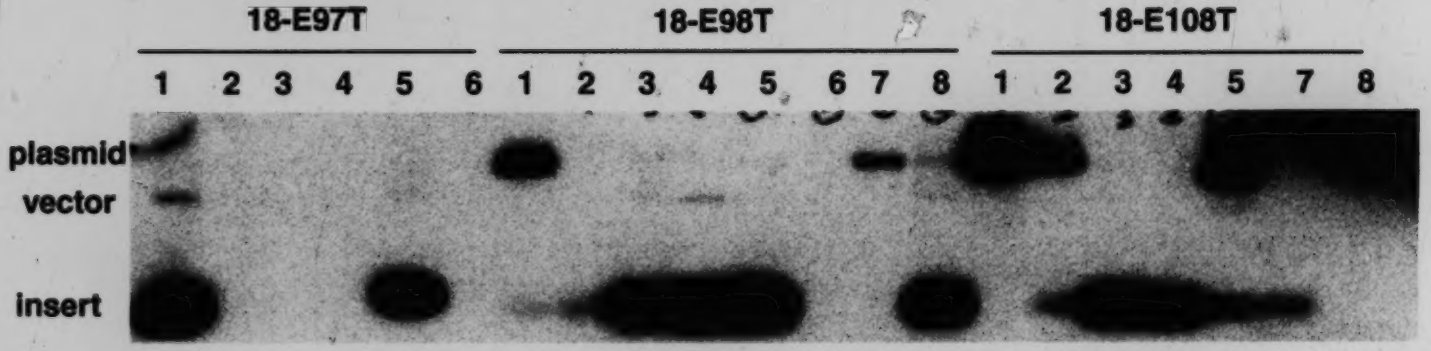
Figure 8. Southern hybridization confirmed the consistency of the insert revealed by polyacrylamide gel electrophoresis (Fragment 18).

(A). Polyacrylamide gel electrophoresis of the ligated fragment 18. The plasmids were cut with EcoRI. 14/24 colonies contain an insert with a size of 210 bp which was consistent with the original reamplified band.

(B). To confirm that all the plasmids containing a similar size of insert were actually the same, a Southern blot with all the EcoRI digested plasmids was hybridized to one of them, insert 18-108-6 (the insert obtained from the No.6 colony containing the recombinant plasmid derived from the band of fragment 18 which was cut from the tumor E108T). The pattern of Southern signals was consistent with that of polyacrylamide gel electrophoresis (see the text).


18-E97T
18-E98T
18-E108T
1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
1 2 3 4 5 6 7
3.9kb
380bp
300bp
210bp

(A)


18-E97T
18-E98T
18-E108T
1 2 3 4 5 6 1 2 3 4 5 6 7 8 1 2 3 4 5 7 8
plasmid
vector
insert

(B)

hybridized to 18-E97T-1. 18-E97T-2 appeared to have a different insert from that of 18-E108T-6 and this was confirmed by Southern hybridization. Although 18-E98T-1 appeared to be of similar size as 18-E108T-6, Southern hybridization did not detect a hybridization signal at the insert position but at the undigested plasmid position. Probably, 18-E98T-1 contained the insert but the plasmid was not cut due to the poor quality of the plasmid preparation. Inserts that were present in most or all candidate colonies were regarded as representative of the original DD-fragment and used to probe Northern blots while the corresponding plasmid was used for DNA sequencing. As an example, we used DNA probe 18-E108T-6 which was derived from fragment 18 in tumor E108T and found to be identical to most inserts derived from fragment 18 based on Southern hybridization to probe Northern blots in order to detect the expression of fragment 18 in tumors, plasmid DNA of this probe was used for DNA sequencing. Plasmids containing inserts derived from fragments 7 and 8 appeared to have inserts with different sizes after EcoRI digestion (data no shown) due to unknown reasons, and it was therefore hard to select plasmids for further research. Therefore, no Northern hybridization and DNA sequencing were performed with these two fragments.

III. Northern hybridization

Northern blots containing 2 μg of polyA+ RNA from each of the seven tumors used for the original display, E97T, E98T, E108T, E53T, E171TR, E171TL and E301T were probed with each of the 9 fragments. Five of the

fragments, 2, 9, 11, 16, 20, showed no hybridization signal. Hybridization with fragment 4 resulted in smears on the blot which were hard to interpret (data not shown). Probing with fragments 10 and 19 resulted in a signal in some lanes, however, the pattern differed from those seen on DD-PCR and no differential expression was identified. In DD-PCR gels, fragment 10 was present in a "repressed or tumor suppressor" way and fragment 19 in an "activated or oncogene" way (Figure 9(A,A')). After Northern hybridization, fragment 10 detected two transcripts of 4.1 kb and 2.4 kb expressed in all tumor samples (Figure 9 (B)) with no obvious change of the signal or size in each lane. Fragment 19 detected one transcript of 2.6 kb consistently in all lanes (Figure 9(B')). No significant difference in signal density among each individual lanes could be detected after comparison of the mRNA amounts loaded using actin hybridization.

Fragment 18 showed a perfect "tumor suppressor" pattern in DD-PCR (Figure 10(A)). Although tumor E171TR had lower signal intensity and E301T was dark when seen from the original X-ray film, no bands corresponding to fragment 18 were present in category II tumors. The reamplified products suggested a size of 210 bp for fragment 18 (Figure 10(B)). Subcloned fragment 18 clearly detected a transcript of 2.2 kb in all 3 tumors of category I as predicted by PCR display, but also a weak signal in some category II tumors. There was decreased expression in tumor E53T judged by the amounts of mRNA loaded for each sample and a questionable signal detected in E301T (Figure 10(C)). Though the Northern result was not in complete accordance to

Figure 9. Northern hybridization was not consistent with the differential display PCR results (Fragments 10 and 19).

(A). DD-PCR showed a complete "repressed" pattern for fragment 10.

(A'). DD-PCR showed that fragment 19 was completely absent from category I tumors (an "activated" pattern).

(B). Subcloned fragment 10 detected two transcripts of 4.1 kb and 2.4 kb in all tumors, that is, no differential expression as predicted by DD-PCR. The amount of RNA loaded was determined by hybridization to actin.

(B'). Subcloned fragment 19 detected one transcript with a size of 2.6 kb in all tumor samples. Though there was a difference in signal density, it was not significant when corrected for the amount of RNA loaded.

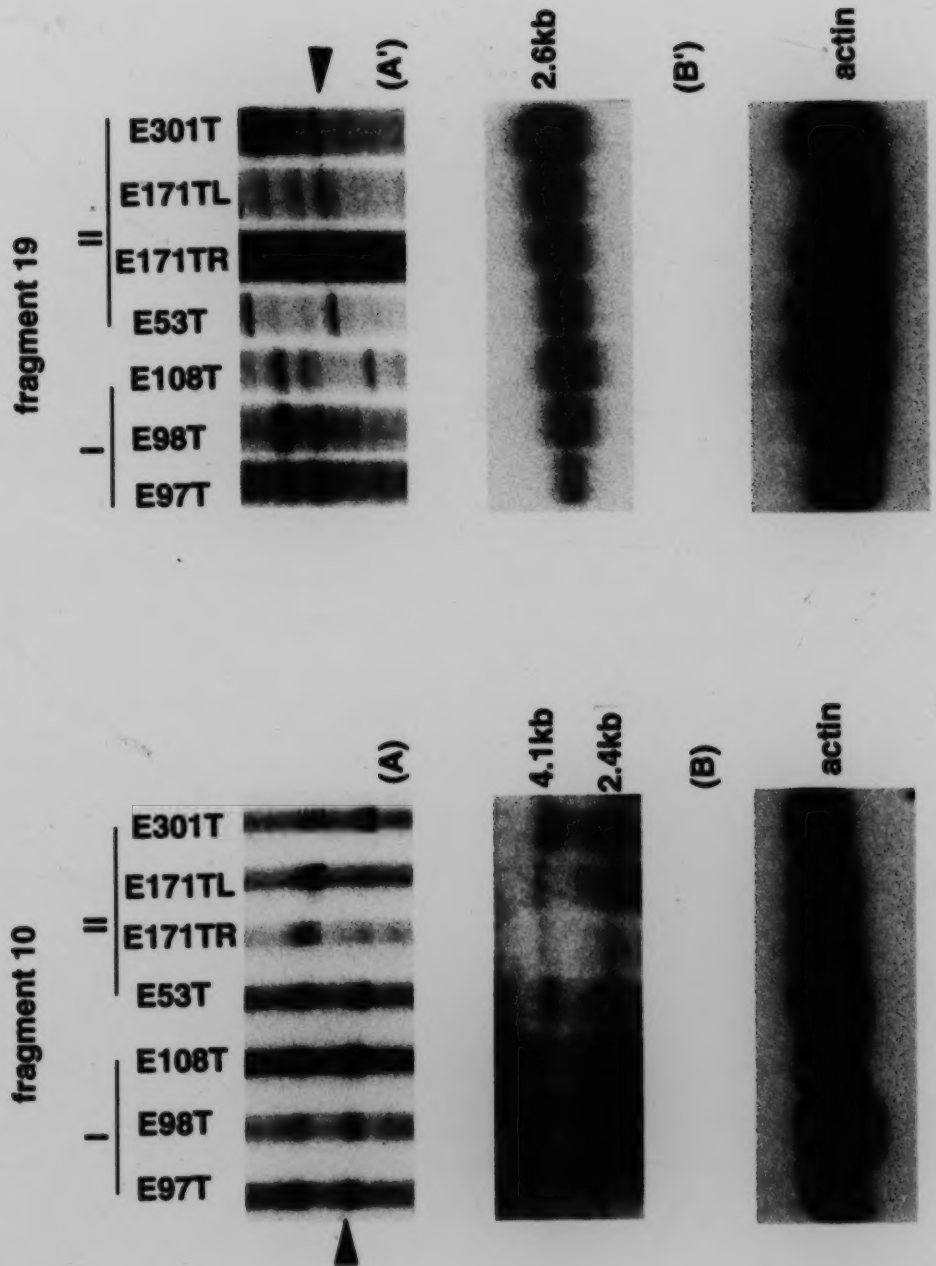

fragment 19
II
I
E301T
E171TL
E171TR
E53T
E108T
E98T
E97T
(A')
2.6kb
(B')
actin
fragment 10
(A)
4.1kb
2.4kb
(B)
actin

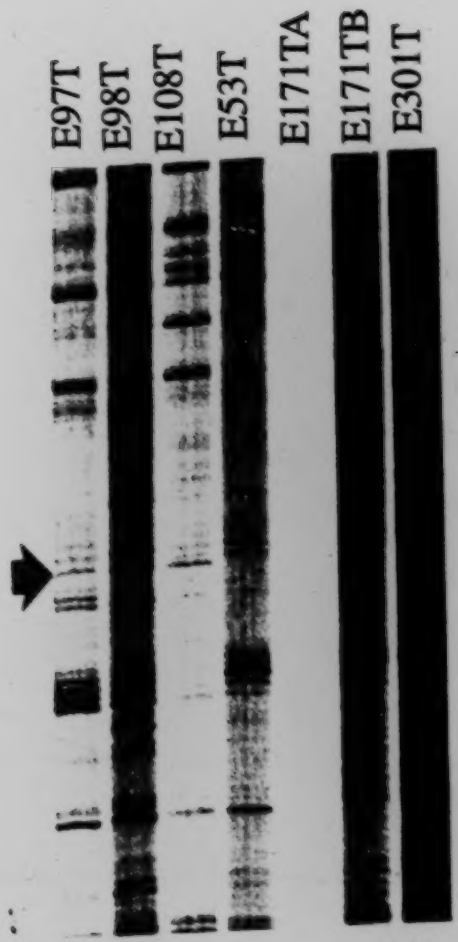
Figure 10. DD-fragment 18.

A). DD-PCR. Fragment 18 had a complete "repressed" display pattern revealed by PCR, that is, it was present in all category I tumors but totally absent from category II.

B). Reamplified products. The band from all three tumors of category I were cut from the PCR gel, eluted and reamplified using the same set of primers and cycle parameters as for the original display. No contamination by other DNAs was detected. The size of the fragment was estimated as about 210 bp.

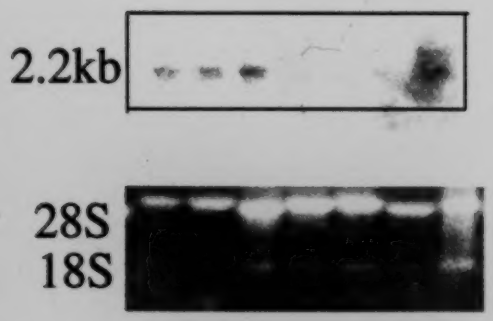
C). Northern hybridization result. Subcloned fragment 18 detected hybridized signals of 2.2 kb in all category I tumors as predicted by PCR, but the signals for category II tumors were not clear-cut. Therefore, Northern hybridization partly confirmed the differential expression of fragment 18. The amount of RNA loaded was determined by 28S and 18S rRNA.

D). DNA sequence. Fragment 18 had no homology to any known human gene listed in GenBank.



(A)

(B)



(C)

5'-TC CTT GAC GCA CAC ATC CAA CTG
CTT ACT TGC CCC CTC CAC TCC ATC
GCC TGT GGA GCA CAT TTT ATT TAG
TTC TAA AGT AGA ACA GTT TGT CTT
TGC CCC CCA CCC TAT GCC CGT TCC
CCA TTA CCA ACT TTC TAG AGT CAC
TCT TGT TTC AGC TCT TTC CCT CTC
AGT ACC CCC TCT CTC CTT GTT TGT
ACC TGC AAA AAA AAA-3'

(D)

that of the DD-PCR, it partly confirmed the differential display of fragment 18 in the tested tumors.

For unknown reasons, there were many difficulties in getting strong signals and clear background for Northern hybridization using the subcloned DD-fragments as probes. Probably the shorter length of these fragments and the relative lower abundance of their mRNA accounted for some of these problems.

IV. DNA sequencing

All 9 fragments, fragments 2, 4, 9, 10, 11, 16, 18, 19 and 20 were sequenced. Both forward and reverse primers were used to get the entire sequence of the fragments, except for fragment 20 for which only forward primer was used. Most of the fragments, 10, 11, 16, 18 and 19, had no similarity to any known human genes listed in Genbank.

In the 272 bp sequence of fragment 2, 38 bp were found to be partly (30/38) identical to human translation initiation factor 5 (eIF 5) mRNA.

87 bp out of the 164 bp sequence of fragment 4 were completely identical to human AP-3 complex delta subunit mRNA.

Both fragments 9 and 20 were highly identical to human mitochondrial genome sequences. For fragment 9, 233 bp of the 314 bp sequence were 100% matched to the 5' end of human mitochondrial DNA. For fragment 20, 100 bp of the 303 bp sequence differed by only one base pair to the mitochondrial DNA at the 3' end.

Fragment 10 had several domains containing TG repeats. No DNA sequences listed in the GenBank were identical to this fragment.

Both fragment 11 and fragment 16 were matched only to non-human DNA sequences.

Fragment 18 (sequence shown in figure 10(D)) was the most interesting fragment in this study since it had a perfect "tumor suppressor gene" expression pattern on the DD-PCR gel and was the only fragment which by Northern hybridization was confirmed (though partially) as a candidate. No homology to any known human gene was detected, however, in the 203 bp of the tested sequence.

V. Chromosome location

To determine the chromosomal location of candidate genes, DD-fragments were used to probe a Southern blot containing of hybrid cell DNAs with each lane having only one human chromosome. Since DNAs were obtained from cell lines containing a human chromosome on a hamster or murine background, cellular DNA from murine, hamster and human genome were used as controls. Fragment 18 was the only candidate which detected a sequence by Northern hybridization, only its chromosomal location was examined and shown to be on human chromosome 3.

CHAPTER IV DISCUSSION AND LATER WORK

I. Selection of tumors and DD-fragments

The main objective of this study was to identify novel Wilms' tumor-related gene(s) by differential display PCR. The putative Wilms' tumor-related gene on the long arm of chromosome 16 was of the most interest in this study. Since in addition to 16q, chromosome 1p and 11p were more likely to be lost or involved in Wilms' tumor development than other chromosomal loci (130,131,136), we chose tumors that did not show LOH in these regions to minimize possible effects of loss of genes on chromosome 1p and 11p. Therefore, by genotype, the tumors from the two categories were known to differ only at 16q. Clinically, the main difference between these two categories was the outcome of the disease (tumor recurred or not). Therefore, any differences in expressed sequences would presumably be due to or be responsible for these differences. In other words, mRNAs expressed differentially between the two categories would hopefully be related to either LOH at 16q or the relapse status of the disease.

In DD-PCR screening, variability in a certain band might not necessarily result from the loss of 16q. Many factors could contribute to the differential display of a fragment. If only one tumor had been selected for each category, the absence of a fragment from one might reflect biological differences between two cells but not necessarily the genetic alteration caused by loss of 16q. For this reason, multiple tumors were included in each category. The presence or absence of a fragment in multiple samples would more likely be significant than

if it occurred in only one tumor. For similar reasons, it was unnecessary to only select fragments with strictly perfect display patterns. Although all test tumors had undergone LOH at 16q, the genetic mutation to the remaining allele was unlikely to have been the same in different tumors and one would predict somewhat variable loss of the representative band. Further, outcome of the disease was unlikely to have been controlled by a single gene and it was reasonable to choose fragments having consistent but not necessarily strict differential patterns as candidates.

II. DD-PCR

The introduction of DD-PCR has provided an improved way to study differential gene expression. It has stimulated much work in cancer research. Though very simple and straightforward in theory, this technique is problematic and challenging in practice.

First, the DD-PCR technique may give rise to a high incidence of false positive changes which can be as high as 70% (157). The false positive bands may be artifacts of reverse transcription and/or the PCR reaction. They can also be derived from contaminating cellular DNA during RNA preparation. In the first several DD-PCR screening experiments of this study, PCR amplification of total RNA without reverse transcription was performed as a control to eliminate the possibility that differential bands might be amplified from contaminating chromosomal DNA. No amplified signals were seen in these control lanes, suggesting little contamination by cellular DNA. Such control reactions were not

used for later DD-PCR screening and this turned out to be a shortcoming of this study based on our later results. Dr. Liang (158) has found out that contaminating DNA did not amplify well after reverse transcription, possibly because the minimal residual amount of DNA could not compete efficiently for dNTPs with cDNAs after reverse transcription. Because we did not carry out control reactions for all experiments, we could not be sure that all the differential fragments obtained in this study were derived from RNA rather than cellular DNA. Particularly, a large part of fragment 9 (233 bp/ 314 bp) was found to be 100% homologous to human mitochondrial DNA, suggesting that it might be a part of the human mitochondrion genome, which indirectly supported the speculation that contaminating DNA could affect the result of differential display screening. One solution to minimize false positives would be to use polyA+ RNA instead of total RNA as the template for reverse transcription. Through the purification procedure for polyA+ RNA preparation, contaminating DNA are greatly eliminated. Another method might be to run duplicates or to repeat the amplifications in which putative differential fragments were identified. Only fragments whose pattern is reproducible would then be considered as candidates. Choosing total RNA as template for reverse transcription, however, was done to conserve experimental materials, since the amount of the tumor tissues was very limited. In fact, repeat experiments starting from cDNA synthesis were performed for the first several DD-fragments to check the reproducibility of differential display. Most displays were highly reproducible,

therefore, running duplicates was eliminated from later experiments, although this may have, in retrospect, adversely affected the results.

The second potential problem of DD-PCR is the low reproducibility of differential expression when tested on Northern blots. In this study, only one of nine candidate fragments was partly confirmed by the differential expression of Northern blot. The high incidence of false positive bands as discussed above is certainly one possible explanation. If the candidate fragment was actually derived from contaminating DNA, there would be an extremely low probability of detecting any homology in an mRNA pool. A third possible explanation for the failure of confirming differential expression at the RNA level is that DNA recovered from seemingly unique bands may have contained additional products. Though there are reports showing that one band represents only one gene (159), other publications have reported that a single band displayed by DD-PCR could be composed of more than one cDNA fragment (160,161). This phenomenon is exemplified by the multiple bands seen after reamplification of fragments 7 and 8 in this study (Fig. 5). In the DD-PCR gel, fragments 7 and 8 both appeared to be a single band in each individual tumor lane with a perfect "oncogene" pattern. After reamplification of the original DD-fragments, however, more than one reamplified band existed which suggested contamination by adjacent bands during excision. These contaminating cDNAs could have been copurified with the desired differentially expressed cDNAs of fragments 7 and 8. Though a predominant reamplified band could be distinguished and was assumed to be candidate fragments 7 and 8, no signal was detected on the

Northern blot. The Southern blot confirmation procedure employed in this study could not identify these "false" inserts if a false insert itself was chosen as a probe. To get around this possible complication, a method to screen cDNAs generated by DD-PCR using Northern blot affinity capturing was proposed by Dr. Li et al (161). Instead of screening multiple recombinants for the desired fragment, they use Northern blots to affinity capture the cDNA fragments. If the initial "unique" DD-fragments contains more than one cDNA, several transcripts should be displayed on the Northern blot. The cDNA fragment of interest, which revealed differential mRNA expression, was then recovered from the membrane and cloned. The difficulty in detecting Northern signals could also be due to a low abundance of the corresponding mRNA. As found in this study, many bands of candidate fragments had a much lower density than those of non-candidates. For instance, fragment 18 was not visible in all category II tumors on the DD-PCR gel. Since the band in category I tumors was very faint, the normal abundance of this mRNA may be very low.

Many candidates were found to be non-homologous to any known human genes (5/9 in this study). On one hand, they may represent novel genes. On the other hand, it is possible that they are not novel but are unmatchable to any known sequences due to the incomplete sequence data for those known genes. To obtain the whole sequence of the mRNA, it has previously been necessary to screen cDNA libraries. A modified "walking" DD-PCR method was designed as an alternative to the time-consuming library screening (162). In brief, this modification applies the general strategy for a regular DD-PCR, but using

different primers. For the first round of DD-PCR screening, the 3' primer containing polyT anneals to the 3' end of mRNA. After the identification and sequencing of a candidate fragment, a new 3' primer which can anneal to the 5' end of this known DD-sequence is designed for the second round of reverse transcription and PCR. Thus the start site of the DD-PCR moves to the 5' end of the corresponding fragment. After several rounds of walking, an additional 5' sequence as long as 1 kb can be obtained.

Despite these technical limitations, DD-PCR is a very promising technique in studying gene expression. It has many advantages: 1) Only PCR and DNA sequencing gel electrophoresis are required in the screening section of the strategy, so candidates can be identified, isolated and confirmed within one or two weeks. It is thus far more rapid and effective in finding differences than other currently used methods. 2) Usually 2 μg of total RNA or 0.1μg of polyA+ RNA is sufficient for the screening of all mRNA species in a cell type at least once. The application of PCR makes it a much more sensitive method in identifying differential expression of mRNAs with low abundance. 3) Since multiple samples can be compared in parallel in a single reaction, it is unique in identifying both over-expressed and suppressed genes simultaneously.

Though this study has not demonstrated the effectiveness of DD-PCR technique in identifying disease related genes, it has supported its feasibility in the study of gene regulation.

III. Wilms' tumor

Wilms' tumor is a heterogeneous tumor. A variety of genetic alterations may be involved in the various subtypes or different aspects of tumorigenesis. Further, in regards to tumor recurrence or relapse, the delineation of prognostic factors is complicated, since the significance of prognostic markers changes with improvements in cancer therapy. The extent of disease at the time of diagnosis has always been an important prognostic criterion. Tumors invading outside of the kidney or with metastatic spread have a lower surgical resectability than those restricted to the kidney. The histological appearance is also an important determinant of prognosis. Tumors composed predominantly of differentiated cell types such as stroma or epithelia have a strong tendency to present with a lower stage (163) thus being associated with a better outcome. Tumors of higher stage often involve distant sites and thus could be predicted to have a higher rate of treatment failure. Favorable versus unfavorable histology, as classified by the NWTS criterion, are also strong prognostic indicators. The two-year survival rate for tumors having favorable histology is about 85%, while for those having unfavorable histology, the rate is as low as 55%. With the introduction of chemotherapy in the 1960s, the sensitivity to chemotherapy and hence the responsiveness to therapy became an increasingly important determinant of outcome. As defined by NWTS-3, unfavorable histology refers only to the anaplastic form of Wilms' tumor. The poor outlook for this subtype appears to not be due to its aggressiveness (ie. invasiveness) but to its resistance to most

current therapies. The presence of anaplasia has been the strongest prognostic factor to date.

Molecular prognostic markers such as chromosomal hyperdiploidy or rearrangements have been found to be associated with adverse outcome. Patients whose tumors contained complex rearrangements had poorer survival than those with diploid or hyperdiploid DNA content but without complex chromosomal rearrangements (164). The presence of hyperdiploidy and complex rearrangements was also significantly correlated with the anaplastic histology as is the presence of p53 mutation.

The finding of LOH for 16q in 20% of Wilms' tumors and the apparent association with relapse indicate a possible prognosis-determining gene located in this chromosomal region. The purpose of this study was to identify a DD-fragment, using the selection criteria of LOH 16q and relapse, which was expressed in a repressed or tumor suppressor gene pattern. Fragment 18 which was partly confirmed to have a repressed pattern of expression in the LOH 16q case is clearly not the sought-after candidate since it is located on human chromosome 3, but it can not be eliminated as a tumorigenic-related gene. For example, it may be regulated by the major prognostic gene(s) on chromosome 16q in Wilms' tumor development. Determination of its relation to Wilms' tumor development and prognosis would require verification in additional Northern expression studies which would be extended to include more Wilms' tumors with or without recurrence.

IV. Future work

If this study were pursued further, initial work would include the identification and characterization of additional differentially expressed mRNAs. Additional arbitrary primers would be used to generate new DD-PCR gels. PolyA+ RNA instead of total RNA would be used as template of reverse transcription and control reactions without reverse transcription would be performed to minimize the possibility of false positive results derived from contaminating cellular DNAs. No matter whether polyA+ RNA or total RNA is used for DD-PCR, duplications for the DD-PCR reactions which revealed candidate fragments would decrease the number of false positive candidates. It is reasonable to assume that identification of an increased number of DD-fragments will increase the chances of identifying the desired gene(s).

For the fragments identified in this study, fragments 2, 4, 9, 11, 16, 20, which failed to give hybridization signals on Northern blots, determination of the chromosomal location might be helpful in determining if they could be significant candidates. If any of them were located on chromosome 16, they would potentially be of interest. One could still proceed to cDNA library screening to facilitate further characterization of the gene and its possible involvement in Wilms' tumorigenesis. Assaying expression in more tumors with similar characteristics to those used in this study, like genotype on chromosome 1p, 11p, 16q and relapse status, might be helpful in determining if they are really related to the Wilms' tumor phenotype.

In the end, all identified and characterized genes would have to be analyzed in tumors to confirm their involvement in Wilms' tumor development.

SUMMARY

DD-PCR technique has been applied in Wilms' tumor study trying to identify novel Wilms' tumor related genes. Thirty DD-PCR screening reactions have yielded twenty-three fragments which are differentially distributed in seven tumors of two categories. Nine of them were tested of their DD-patterns by Northern blot hybridization, only one fragment was partly confirmed of its differential display. DNA sequencing of five fragments revealed no homology to any known human genes, while two of them were highly homologous to human mitochondrion genome. Further work needs to be done to identify more candidate fragments and to characterize their involvement in Wilms' tumor development.

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